POLYSACCHARIDE COMPONENTS OF ALFALFA

by

MAHBOOB UDDIN, M.Sc.

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TO MY PARENTS

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INTRODUCTION

(SECTION I)

Polysaccharide components of alfalfa

Alfalfa (*Medicago sativa* Var Ranger) also known as lucerne is one of the major legume crops of southern counties of England. It belongs to the same family as beans, peas and clover. It is the best fodder plant as it provides a rich source of nitrogen and calcium.

The polysaccharides present in plants can be divided into two groups:- (a) reserve polysaccharides, and (b) cell wall polysaccharides. Alfalfa contains small amounts of reserve polysaccharides. Unlike grasses, it does not contain any fructan and only a small amount of water soluble glucan has been reported to be present in alfalfa. It contains cellulose, hemicelluloses and pectins as cell wall polysaccharides (1).

The first detailed structural investigation of polysaccharides of alfalfa was carried out by Myhre and Smith (2)(3). By extracting alfalfa with aqueous sodium hydroxide they isolated a polysaccharide fraction which contained the residues of L-arabinose (12·0%), D-Xylose (67·3%), D-galactose (8·1%), D-glucose (8·1%), L-rhamnose (4·5%), D-galacturonic acid and 4-O-methyl-D-glucuronic acid. Since this so-called hemicellulose was obtained by extracting the hay directly with alkali without prior removal of pectic materials, it is probable that this polysaccharide preparation was a mixture of pectic materials and the polysaccharides of xylan group. The presence of both of these polysaccharides in alfalfa was indicated earlier (1) and has been confirmed by further

investigations (4)(5). Partial acid hydrolysis of the polysaccharide preparation gave five acidic oligosaccharides:-

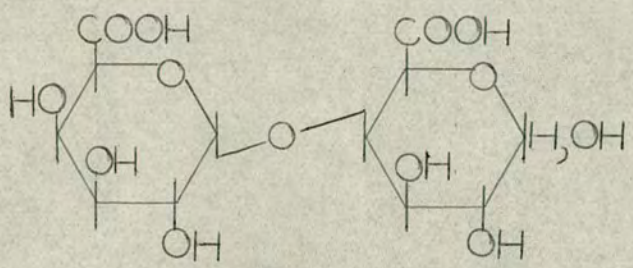
1. 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylose
2. O-(4-O-methyl-α-D-glucopyranosyluronic acid)-(1→2)-O-β-D-xylopyranosyl-(1→4)-β-D-xylose
3. 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose
4. 6-O-(β-D-glucopyranosyluronic acid)-D-galactose
5. 2-O-(α-D-glucopyranosyluronic acid)-D-xylose.

In the light of later investigations it is evident that the oligosaccharides 1 and 2 might have arisen from hemicelluloses of xylan group (5) and oligosaccharide 3 from pectic acid (4), whereas oligosaccharide 4 has been identified among the products of hydrolysis of pectic material of alfalfa, during the present investigation.

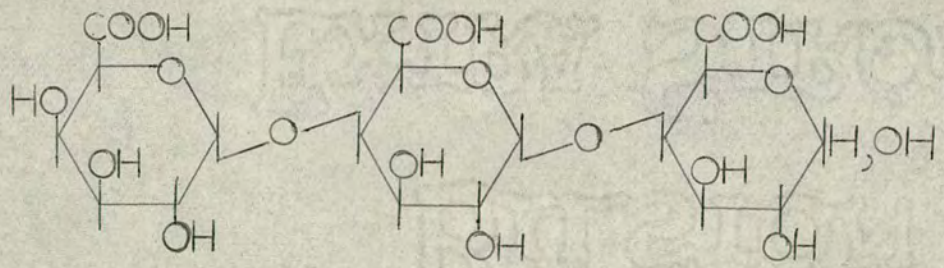
Aspinall and Fanshawe (4) obtained a number of polysaccharide fractions by a stepwise extraction of alfalfa. Extraction with hot ethanol-water (4:1) removed colouring matter and soluble sugars among which D-glucose, D-fructose, sucrose and raffinose were identified. The cold- and hot-water extracts contained complex mixtures of acidic polysaccharides which were contaminated with inorganic material and protein. Successive extraction with hot ammonium oxalate solution gave ammonium pectate which was purified through calcium salt and found to be homogeneous by diethylaminoethylcellulose chromatography. Extraction of the residue with lime water gave some arabinan rich polysaccharide.

Ammonium pectate so obtained was found to contain residues of D-galacturonic acid (50%), L-arabinose, D-galactose, L-rhamnose, and

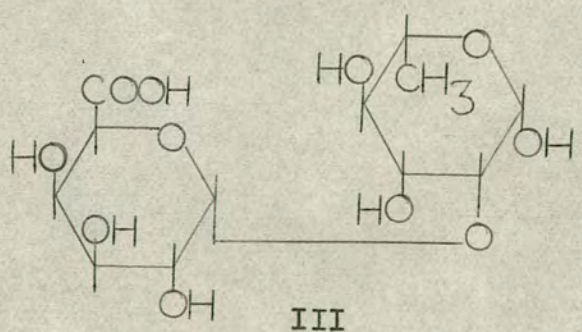
traces of L-fucose, 2-O-methyl-L-fucose and 2-O-methyl-D-xylose. Partial acid hydrolysis of the polysaccharide furnished oligomers of galacturonic acid, i.e., 4-O-(α-D-galactopyranosyluronic acid)-D-galacturonic acid (I) and O-α-D-galactopyranosyluronic acid-(1 → 4)-O-α-D-galactopyranosyluronic acid-(1 → 4)-D-galacturonic acid (II),



I



II

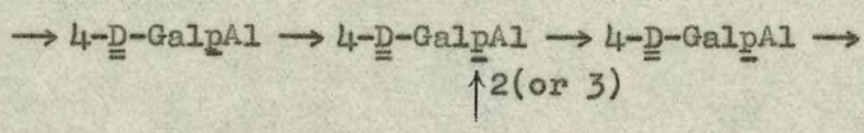


III

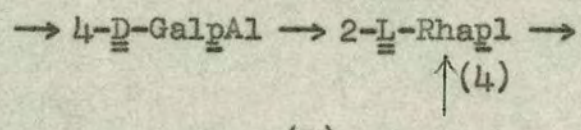
2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose (III), a higher oligosaccharide containing galacturonic acid and rhamnose residues, a disaccharide containing galacturonic acid and L-fucose residues

and another disaccharide, O-(galactosyluronic acid)-D-galactose. The structures of the last three oligosaccharides could not be fully established, but it was proved that some neutral sugars are integral constituents of the pectic material. The aldobiouronic acid, 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose was the same oligosaccharide 3, isolated from partial hydrolysis of the so-called hemicelluloses of alfalfa (3), which polysaccharide preparation presumably also contained pectic material.

Further information about the structure of ammonium pectate was obtained by methylation results. Methanolysis of methylated pectic acid followed by reduction and hydrolysis afforded 2,3,5-tri- and 2,3-di-O-methyl- and 2-O-methyl-L-arabinose, 2,3,4,6-tetra-, 2,3,4-tri- and 2,3-di-O-methyl-and 2- and 3-O-methyl-D-galactose and 3,4-di-O-methyl- and 3-O-methyl-L-rhamnose. From partial hydrolysis and methylation results it was concluded that the polysaccharide consists of 1,4 linked α-D-galacturonic acid residues (A), and L-rhamnose residues are components of the pectic material some of which may act as branch points (B).



(A)



(B)

The methyl ethers of L-arabinose isolated from the methylated polysaccharide showed that some L-arabinofuranose end groups were

present in the polysaccharide. If all arabinose residues were present in the furanose form, the modes of linkage of this sugar were the same as those in the arabinans of pectic materials (6) (structures C-E). The relatively high proportion of D-galactose residues in the polysaccharide present as end groups (F) showed that these are probably linked in some way to the framework of D-galacturonic acid residues.

L-Araf1 → → 5 L-Araf1 → → 5 L-Araf1 → D-Galp1 →
(C) (D) (E) 3↑ (F)

These results however confirmed the view that pectic acids are a complex group of acidic polysaccharides which contain substantial proportion of neutral sugars.

In another series of stepwise extraction of polysaccharides from separated leaves and stems of alfalfa, Aspinall and McGrath (5) have isolated 4-O-methylglucurono-xylans. These hemicelluloses were extracted with dilute alkali, after removing soluble sugars with ethanol-water (4:1) and pectic material with water and solutions of ethylenediamine-tetra-acetic acid disodium salt. Partial hydrolysis of the hemicelluloses gave oligosaccharide 1 and 2 obtained by Myhre and Smith (2) as well as their higher homologues. In addition, the following neutral oligosaccharides were obtained:-

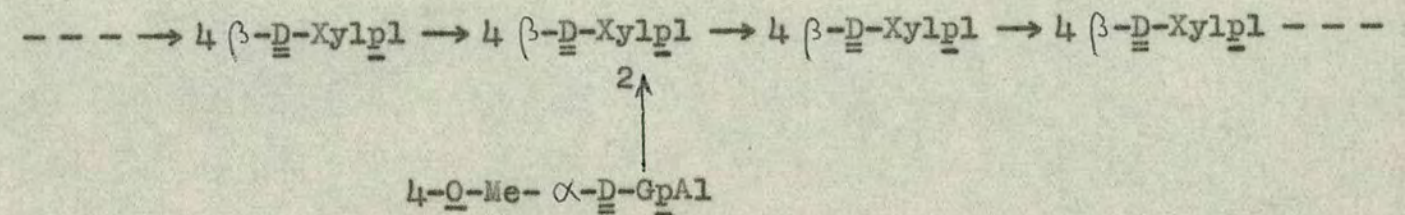
6. β-D-Xylp1 —(→ 4 β-D-Xylp1—)$_n$→ 4 Xylp (n = 0-4)

7. Xylp1 → 3 Rha

8. Xylp1 → 4Xylp1 → 3 Rha.

On the basis of methylation and partial hydrolysis results,

Aspinall and McGrath (5) have shown that alfalfa leaf and stem xylans contain linear chains of 1,4 linked β-D-xylopyranose residues with approximately every ninth residue carrying through C-2 single unit side chains of 4-O-methyl- α-D-glucuronic acid (G) and the main chain also contains occasional rhamnose residues.



(G)

The seeds of alfalfa contain substantial amount of galactomannans. Hirst and Jones (7) have isolated an unusual type of galactomannan from alfalfa seeds by extraction with hot potassium hydroxide solution, while a galactomannan having similar composition and structure to galactomannans from other leguminous plants, has been isolated by Andrews, Hough and Jones (8), by extracting the seeds with hot water.

(SECTION II)

Since this thesis is concerned mainly with the structural investigations of pectins present in alfalfa, a brief introduction to the chemistry of pectic substances is given in this section.

Pectic Substances

Definition of terms involved in pectin chemistry:-

A number of terms which are rather confusing are involved in pectin chemistry. The nomenclature recommended by a committee of American Chemical Society (9) was revised in 1944 (10). According to the revised nomenclature of pectic substances, the following definitions are in use:-

1. Pectic substances: "Pectic substance is a group designated for the complex colloidal carbohydrate derivatives, which occur in or are prepared from plants and contain a large proportion of galacturonic acid units which are thought to exist in a chain like combination. The carboxyl groups of polygalacturonic acid may be partly esterified by methoxyl groups and partly or completely neutralized by one or more bases."
2. Pectinic acids: "The term pectinic acid is used for colloidal polygalacturonic acids containing more than a negligible proportion of methyl ester groups."
3. Pectin: "Pectin or pectins are those water soluble pectinic acids of varying methoxyl content and degree of neutralization which are capable of forming gels with sugar and acid under suitable conditions."

4. Pectic acids: "Pectic acids are those pectic substances which are essentially free from methyl ester groups. The salts of pectic acids are either normal or acid pectates."

Pectic substances occur in almost all plant tissues. They are found in large amounts in soft tissues composed mainly of primary cell walls, while in hard tissues, such as wood, they are present in negligible amount. The middle lamella or intercellular substance of the tissues is composed largely of pectic substances. They also occur in secondary cell walls and in the sap of fruit cells.

The function of pectic substances in plants is not fully understood. It is believed that the pectic substances of the middle lamella are not only responsible for the cementing together of the adjacent cells, but also undergo transformations which cause the softening and eventual maceration of the tissues. Being hydrophilic in nature and capable of taking and holding quantities of excess of water, they may be responsible for the proper hydration of young cell walls.

Pectins have been a very important group of polysaccharides for their property of forming gels (jellies). Generally two types of gels of pectic substances are known. The first type is the conventional pectin-sugar-acid-water gel, which consists of 50% or more of sugar and is formed exclusively through hydrogen bondings. The second type is the ion-bonded gel of low ester pectinic acids or pectic acids formed by the multivalent ions, especially of calcium.

The molecular weights of pectins isolated from different

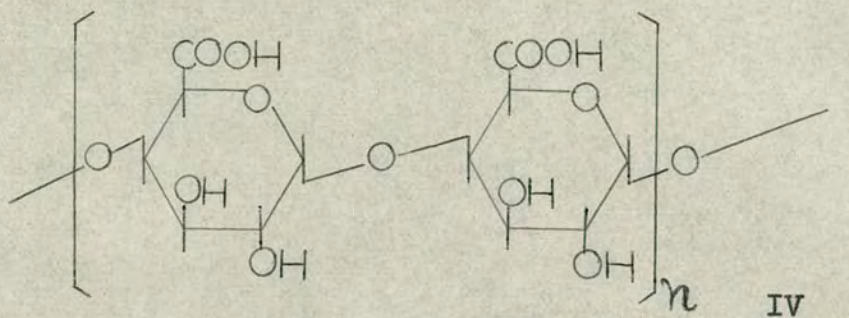
sources and also from the same source but by different extraction procedures vary considerably ranging from about 10,000 to 400,000. This anomaly clearly shows that some degradation of pectin molecule always occurs during extraction. Schneider and co-workers have obtained direct evidence in support of this view, by viscosity measurements of nitrated derivatives of pectins. Schneider and Henglein (11) found that the nitrated pectin of sugar beet slices had molecular weights of 50,000-100,000, while the nitrated derivative of hot water extracted pectin of the same material had molecular weight of 30,000-50,000. In the same way Schneider and Bock (12) found that the molecular weight of nitrated derivative of apple pomace pectin obtained from first extract was much ~~more~~ higher than that obtained from subsequent extracts.

Pectic Triad:- Although the pectic substances are composed mainly of galacturonic acid residues, they always contain varying proportions of D-galactose and L-arabinose residues. The early work on the structure of pectic substances suggested that they consist of three homopolysaccharides - the pectic triad, namely, a galacturonan, a galactan and an arabinan. Some workers have tried to isolate the various homopolymers from pectic preparations, but it is remarkable that all the three homopolymers have never been isolated from a single source. In view of the fact that pectins are very susceptible to degradation under various conditions, as will be discussed more fully later, it is possible that certain homopolysaccharide preparations might have arisen from degradation of pectin molecules during extraction and fractionation. Nevertheless, the work on these homopolymers was very important in establishing

certain aspects of the structural chemistry of pectins. Recent studies on pectins have shown that although homopolysaccharides may be genuine components of some preparations, the major components, which may be isolated under the mildest possible conditions, are heteropolysaccharides.

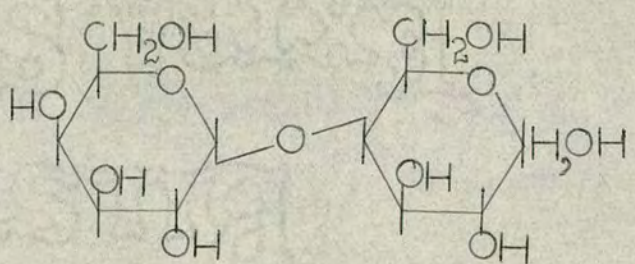
Pectic galacturonans:- In the early investigations, the galacturonans were obtained as degraded polysaccharides since acid hydrolysis was used to remove accompanying galactose and arabinose residues. Levene and Krieder (13) elucidated the structure of galacturonans for the first time. By periodate oxidation studies, they showed that the galacturonan chains consist of either 1 and 4 linked galactopyranosyluronic acid units or 1 and 5 linked galactofuranosyluronic acid units.

Luckett and Smith (14), Hirst (15) and Beaven and Jones (16) elucidated the structures of galacturonans, isolated by acid hydrolysis or methanolysis of pectins from citrus fruits, white lupin seeds, raspberries, strawberries and apples, by methylation studies. Methylation and subsequent oxidation of methyl-2,3-di-O-methyl-D-galacturonoside, obtained as a sole product from the methanolysis of methylated galacturonans, gave 1:4 lactone of 2,3-di-O-methylgalactaric methyl ester. Taking into account the fact that polymers were quite resistant to hydrolysis and had high positive optical rotations, these workers suggested that the galacturonans were composed of chains of 1,4 linked α-D-galacto-pyranosyluronic acid units (structure IV).



IV

Jones and Reid (17)(18) isolated galacturonobiose and galacturonotriose by enzymic hydrolysis of apple pectic acid. The galacturonobiose was shown to be 4-O-(α-D-galactopyranosyluronic acid)-D-galacturonic acid (I, p.3), by reduction to 4-O-(α-D-galactopyranosyl)-D-galactose (V). These results confirmed the earlier proposal



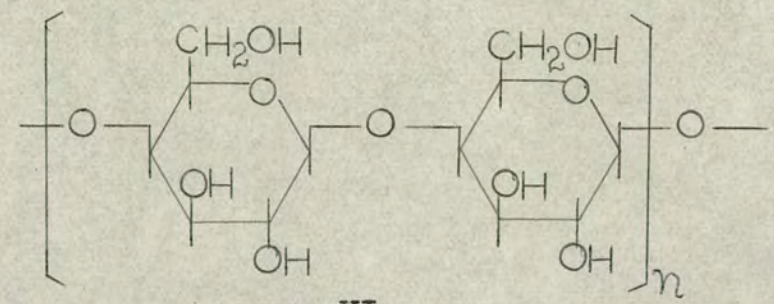
V

that galacturonans consist of 1,4 linked α-D-galactopyranosyluronic acid residues.

Recently galacturonans have been isolated from pectic materials under the mildest possible conditions. Bishop (19) isolated a galacturonan from sunflower heads by extraction with hot ammonium oxalate-oxalic acid solution. More recently Bishop and Zitko (20)(21) have isolated pure galacturonans by fractionation of pectic acids formed on saponification of pectins from sunflower heads, sugar beet, apple and citrus fruits, while Timell and Bhattacharjee (22) have fractionated a galacturonan from pectins present in the bark

of amabilis fir. The galacturonans from sunflower heads (21) and amabilis fir (22) pectic acids were reduced to the corresponding galactans and the methylation studies of galactans showed that galacturonans consist of galactopyranosyluronic acid units linked through 1 and 4 positions in the α- configuration. (Structure IV)

Pectic galactans: Hirst, Jones and Walder (23) for the first time isolated a galactan from the pectic material obtained by alkaline extraction of white lupin seeds. The acidic polysaccharide was removed by precipitation as the calcium salt and accompanying arabinan was partly removed by extraction with 70% methanol. The remaining residues of L-arabinose were removed by subsequent hydrolysis of the polysaccharide with dilute oxalic acid. It should be noted, however, that the resulting galactan may have arisen from the removal of arabinose residues from an arabinogalactan, or from a mixture of galactan and accompanying arabinan. Moreover, the authenticity of galactan is questionable as during alkaline extraction of pectic material, which in light of recent investigation, is very labile to base-catalysed degradation, the large complex molecules of pectic material might have degraded to give the mixture of polysaccharides extracted. From the results of methylation and specific rotation of the galactan, it was shown that the galactan molecule consists of a linear chain of D-galactopyranose residues linked through 1 and 4 positions in the β-configuration (VI).



VI

In some instances, galactans have been found to exist as strictly homopolysaccharide in pectic preparations. A structurally similar galactan has been isolated as a minor component from commercial citrus pectin (24), by precipitating the acidic polysaccharide as insoluble copper salt. Andrews, Hough and Jones (25) have isolated a galactan of the same type from seeds of Strychnos nux-vomica.

Galactans of a complex nature are found in wood. A galactan with 13% uronic acid residues and containing both, galacturonic acid and glucuronic acid was isolated from Norwegian spruce compression wood (26). The neutral part of the polysaccharide was shown to consist of a linear chain of 1,4 linked β-D-galactopyranose residues. A similar complex type of galactan was isolated from beech wood (27).

Besides pure galactans, arabinogalactans have also been shown to be associated with the pectic substances. An arabinogalactan consisting of 1,4 linked β-D-galactopyranose residues with side chains of L-arabinofuranose residues linked through 1 and 5 positions to the galactan framework at 3 position has been isolated from defatted soy-bean flour (28). Another arabinogalactan of a

more complex nature has been isolated from Centrosema plumari seeds (29).

Pectic arabinans: Hirst and Jones have isolated a number of arabinans from pectins of peanuts (30)(31)(32), sugar beet (33), citrus fruits (34), and apple (35), and carried out structural investigations. The same type of arabinans have been isolated from sugar beet pectin (36)(37). In all cases methylation and subsequent hydrolysis of the methylated polysaccharide gave equimolar proportions of 2,3,5-tri- and 2,3-di-O-methyl- and 2-O-methyl-L-arabinose. The ease of hydrolysis and a high negative specific rotation suggested that all arabinose residues were in furanose form and that the configuration was α. These results show that these polysaccharides contain the structural features (C), (D) and (E) (page 5) and the repeating unit may be either (H) or (I)

(H) (I)

These elucidations have been supplemented by the isolation of oligosaccharides, 5-O-L-arabinofuranosyl-L-arabinofuranose and 3-O-L-arabinofuranosyl-L-arabinose, by graded hydrolysis of sugar beet arabinans (38).

Recently arabinans of pectic type have been isolated from mustard seeds (39), under conditions, which do not allow any appreciable degradation of the polysaccharide. The results of

methylation analysis show the similarity between mustard seed arabinans and other pectic arabinans. Hydrolysis of the methylated polysaccharide gave 2,3,5-tri-O-methyl-L-arabinose, 2,3,-di-O-methyl-L-arabinose, 2-O-methyl-L-arabinose and L-arabinose in the molar proportion of 10:5:9:1·7, which shows that the polysaccharide is highly branched. Although the methylation analysis gave no indication as to the ring size of the arabinose units in the polysaccharide (other than at the non-reducing end groups), the ease of hydrolysis suggests that these units are in furanose form. These are joined by 1,5 linkages with a considerable proportion of 1,3 branch linkages. The presence of arabinose in the hydrolysate of the methylated polysaccharide indicates that certain of the branched units are also linked through position 2. The same type of arabinan has been isolated from the aqueous extracts of maritime pine (40), but this polysaccharide contains a small amount of galactose as non-reducing end groups.

Complex nature of pectins: Structural investigations of pectic substances have led to the view that some neutral sugars are integral constituents of this complex group of acidic polysaccharide. In the early investigations D-xylose was reported to be a constituent sugar of pectins from orange (41) and flax (42), while D-fucose (rhodeose) that of ramie bast pectin (43). McCready and Gee (44) found that purified pectinic acids from several fruits and vegetables gave on hydrolysis galacturonic acid with varying amounts of galactose, arabinose, rhamnose and xylose. These sugars were also constituents of the pectic substance isolated from fresh water green algae Nittella translucens (45). The same sugars

along with 2-O-methyl-D-xylose and 2-O-methyl-L-fucose were obtained from the hydrolysis of sisal flash pectin (46). Pectic substances present in lucerne (4), soy-beans (47)(48) and lemon peel (49), which were resistant to attempted fractionations were found to contain all these neutral sugars along with L-fucose. Neutral sugar residues were found in all the polysaccharide fractions when Neukom and co-workers (50) tried to fractionate sugar beet pectin on diethylaminoethyl-cellulose, whereas a known mixture of homopolysaccharides was successfully fractionated into neutral and acidic polysaccharides on the said ion-exchange column.

L-Rhamnose was the first neutral sugar conclusively shown to be an integral constituent of acidic polysaccharide components of pectic material. The evidence was obtained by the isolation of aldobiouronic acid, 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose (III, p.3) formed by the partial hydrolysis of, (a) pectic type acidic polysaccharide from grapes (51), (b) lucerne pectic acid (4), (c) pectins present in the bark of amabilis fir (22), (d) acidic polysaccharides of soy-beans (47)(48), (e) commercial citrus pectin (24), and (f) pectins extracted with cold water from lemon peel (49). More recently, the fact that L-rhamnose is a constituent neutral sugar of pectins has been further demonstrated by the isolation of a number of higher oligosaccharides containing galacturonic acid and rhamnose units by the partial hydrolysis, acetolysis or enzymolysis of the pectins obtained by different sources. In the table below, oligosaccharides 1 and 2 were obtained by partial hydrolysis, 3 and 4 along with 2 by acetolysis and oligosaccharide 5 by enzymic hydrolysis.

S No.	Oligosaccharide	Pectins		
		Soy-bean cotyledon meal	Soy-bean hull	Lemon peel
1	GalpA1 $\xrightarrow{\alpha}$ 4GalpA1 $\xrightarrow{\alpha}$ 2Rha		+	+
2	GalpA1 $\xrightarrow{\alpha}$ 2Rhap1 $\xrightarrow{\beta}$ 4GalpA1 $\xrightarrow{\alpha}$ 2Rha	+	+	+
3	GalpA1 $\xrightarrow{\alpha}$ 2Rhap1 $\xrightarrow{\beta}$ 2Rha	+	+	+
4	GalpA1 $\xrightarrow{\alpha}$ 4GalpA1 $\xrightarrow{\alpha}$ 2Rhap1 $\xrightarrow{\beta}$ 2Rha	+		
5	GalpA1 $\xrightarrow{\alpha}$ 2Rhap1 $\xrightarrow{\beta}$ 4GalpA			+
	References	(47)	(48)	(49)

Table I

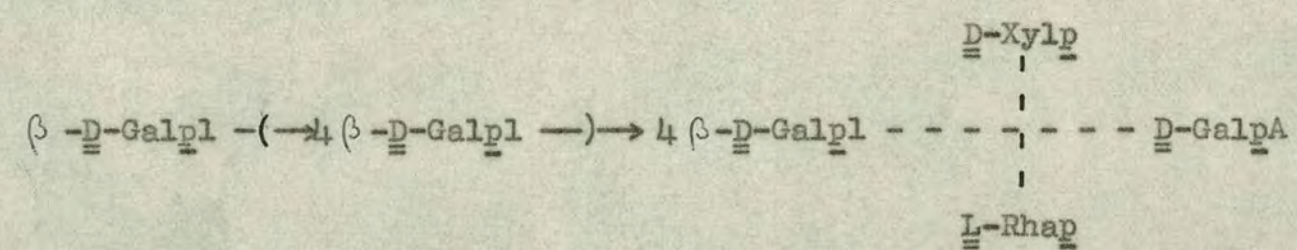
These results together with the results of methylation of the acidic polysaccharides clearly show that in the molecule of pectin the main chain of 1,4 linked galactopyranosyluronic acid units is interrupted by occasional rhamnose residues, some of which constitute the branch points, and that at least two rhamnose units are adjacent in parts of the structure, (J).

GalpA1 → 4GalpA1 → 4GalpA1 → 2 Rhap1 → 4GalpA1 → 2Rhap1 → 2Rhap1

(J)

As yet there is no direct evidence for a linkage between arabinose or galactose units and galacturonic acid units but these

sugars seem to be the constituents of pectic acids as they are not removed even by exhaustive fractionation of the polysaccharide. The methylation studies of pectic acids from various sources have shown that a large proportion of residues of these sugars exist as non-reducing end groups. In some instances, however, these sugar residues are found to be attached in structural units with linkages similar to those in the respective arabinans and galactans of pectic preparations. For example, in lucerne pectic acid (4) galactose residues were shown to be present mainly as non-reducing end groups, whereas arabinose residues were linked in a branched structure as in arabinans of pectic type, while in sisal pectic acid (46) arabinose residues predominantly as end groups were indicated. Pectic acid from bark of Amabilis fir (22), on the other hand, was shown to contain both the neutral sugars mainly in the form of end groups. In contrast, 4-O-(β-D-galactopyranosyl)-D-galactose and the corresponding higher homologues have been isolated as partial hydrolysis products from the acidic polysaccharides from soy-bean (47)(48) and also from commercial citrus pectin (24), showing the presence of galactan chains in these pectins. Enzymic degradation of pectic material from soy-bean (47) has also given a higher oligosaccharide, not fully characterised, in which chains of 1,4 linked galactopyranose residues are linked either directly or through xylose or rhamnose to single unit galacturonic acid, (K).



(K)

Although glucuronic acid has not been identified as a constituent sugar of pectins by hydrolysis of the polysaccharide, recently comparatively small amounts of glucuronic acid containing aldobiouronic acids have been identified among the products of partial hydrolysis of some pectin preparations. Two aldobiouronic acids, 6-O-(β-D-glucopyranosyluronic acid)-D-galactose and 4-O-(β-D-glucopyranosyluronic acid)-L-fucose have been isolated, in comparatively low yields, by the partial hydrolysis of lemon peel pectin (49) and acidic polysaccharides of soy-beans (47)(48). The latter polysaccharide has given another acidic oligosaccharide, 4-O-(β-D-glucopyranosyluronic acid)-D-galactose. The exact structural significance of these oligosaccharides, however, is not known.

Recently a xylose containing acidic oligosaccharide, 3-O-β-D-xylopyranosyl-D-galacturonic acid has been isolated from enzymic degradation of acidic polysaccharides from soy-beans (47) and also from lemon peel pectin (49), which shows that xylose is also an integral constituent of some pectins.

Degradation of pectic substances: Pectins are very susceptible to degradation under various conditions. They are easily degraded by acids, alkalis, enzymes and even in neutral solutions. This property of pectic substances presents a number of problems during extraction and fractionation of the polysaccharide. Also, of course, degradation procedures such as partial hydrolysis and enzymolysis have been used to positive advantage in the structural investigations of pectins.

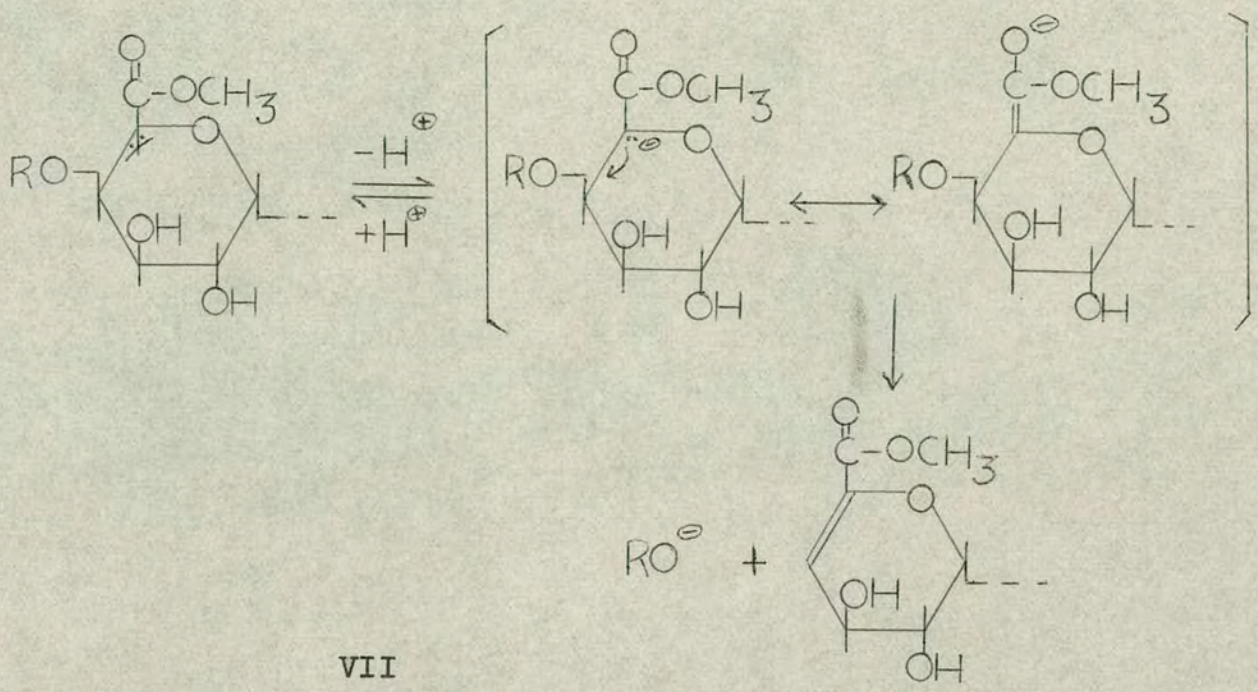
Acid degradation: Very weak acid solutions are capable of removing arabinofuranose residues present in the pectic substances, and therefore care should be taken not to use hot acidic solutions for extraction of the polysaccharide. In contrast, partial acid hydrolysis is used to positive advantage in structural investigations, to isolate acidic oligosaccharides such as 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose.

Alkaline degradation: In the absence of oxygen, polysaccharides undergo alkaline degradation which begins at the reducing end and proceeds in a stepwise manner along the sugar chain. Pectic substances containing 1 $\longrightarrow$ 4 linked galacturonic acid residues are degraded by this peeling process giving corresponding saccharinic acids (52). This type of degradation causing a small decrease in the molecular weight of the pectin molecule is not readily detected by the methods such as viscosity measurements. In this type of degradation, the glycosidic linkages remote from the reducing end of the chain are not effected.

Vollmert (53) has shown that alkaline saponification of pectin at 20° effects the splitting of a few glycosidic linkages within the chain. He also found that pectins are easily degraded in alkaline solution while pectic acids are fairly resistant, and thus it was concluded that the degree of depolymerisation is a function of extent of esterification of pectin. Launer and Tomimatsu (54) have established that during alkaline saponification of pectin at 25°, for approximately eighty de-esterifications which occur, one glycosidic linkage is split. Hatanaka and Ozawa (55) have recently

found that temperature and pH of the solution plays an important role in the alkaline saponification of pectins. They have determined the suitable conditions for saponification of pectins without any appreciable depolymerisation of the molecule.

Neukom and Deuel (56) have studied the degradation of citrus pectin by alkaline saponification at various temperatures, measuring the viscosity of the resulting sodium pectate solution. They found that by selecting the proper saponification temperature, the viscosity of sodium pectate produced can be varied at will. Similar results were obtained with the glycol asters of pectic acid. It was found that the de-esterified sodium pectate formed was not depolymerised by alkali under these conditions, but athigher temperatures was further degraded by the usual stepwise alkaline degradation from the reducing end. The remarkable instability of the esters of pectic acid, in alkaline solution, is explained assuming a β-dealkoxylation mechanism (56). According to this mechanism the glycosidic linkage in the β-position to the ester carbonyl group of pectin is cleaved, following the removal of the activated hydrogen at $C_{(5)}$ and the formation of a double bond between $C_{(4)}$ and $C_{(5)}$. The non-esterified carboxyl group at $C_{(6)}$ is not sufficient to activate the hydrogen at $C_{(5)}$, in alkali, and therefore the splitting of glycosidic bonds, as shown below, does not occur in sodium pectate.



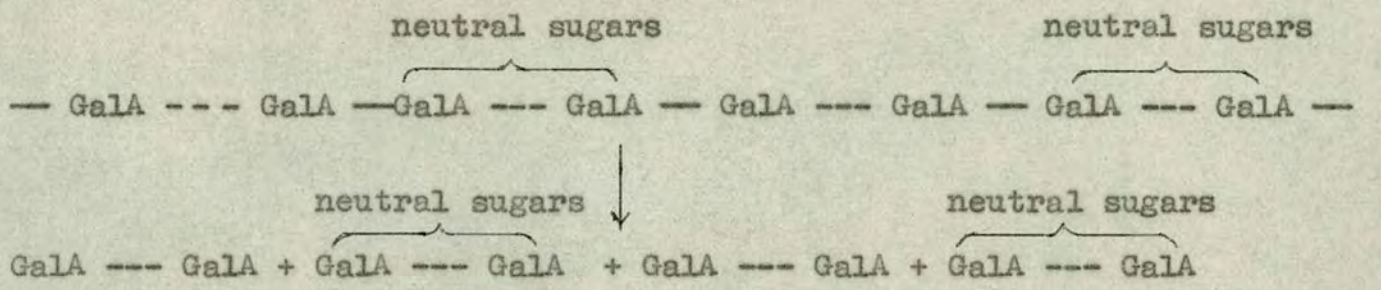
VII

(R is the remaining portion of the molecule of polysaccharide)

Degradation in neutral solutions: Pectic substances are labile to degradation in hot solutions of phosphate buffers (57). Neukom and co-workers (58) have found that, as in the case of alkaline degradation, β-elimination and cleavage of glycosidic bonds of the polysaccharide occurs, by hot buffer treatment at pH 6·8. This was indicated by the reduction of relative viscosity of the resulting solution with the increase of temperature, and by an increasing absorption at 235 mμ, characteristic of α,β-unsaturation.

By treating apple pectinic acid with hot phosphate buffer at pH 6·8, Barrett and Northcote (59) have isolated two components of polysaccharide completely separable by zone electrophoresis and by Sephadex gel filteration. The high molecular weight component was shown to contain a large proportion of neutral sugars, while the low molecular weight component was predominantly composed of galacturonic acid units. They suggested that the pectinic acid

molecule which is probably composed of large arabinofuranose blocks attached to the galacturonosyl chains is degraded into two fragments: one containing predominantly galacturonic acid residues and the other mainly neutral sugars.



(L)

(GalA --- GalA denotes 20-50 galacturonic acid units)

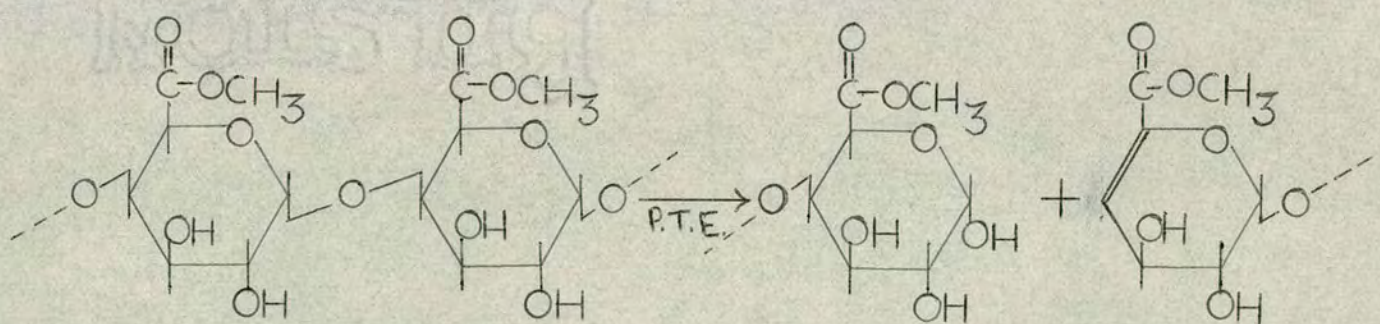
Enzymic degradation: Pectic enzymes can be classified into three groups. The first group consists of pectinesterases which de-esterify the methyl esters of pectins. The second group includes the polygalacturonases which cleave the glycosidic linkages and the third group comprises the pectintranseliminases which degrade the pectin by a β-elimination type of mechanism.

Fungal polygalacturonases cause the graded hydrolysis of pectins producing galacturonic acid, galacturonobiose, galacturonotriose and galacturonotetraose (60). It has also been suggested that these enzymes are mixtures of three or more components, one of which hydrolyses pectin to galacturonobiose and galacturonotriose, while another hydrolyses these to galacturonic acid (61)(62). Yeast polygalacturonase hydrolyses pectic acids producing galacturonic acid, di-, tri- and tetra-galacturonic acid (63)(64). Demain and Phaff (65)(66) have studied the action of this enzyme on a series of oligogalacturonides. They found that galacturonotetraose gives galacturonotriose and galacturonic acid, galacturo-

notriose gives galacturonobiose and galacturonic acid, while galacturonobiose is not at all attacked by the enzyme.

Acidic oligosaccharides containing neutral sugar residues have also been isolated by enzymic degradation of pectic substances. Aspinall and Craig (49) have isolated O-α-D-galactopyranosyluronic acid-(1 → 2)-O-β-L-rhamnopyranosyl-(1 → 4)-D-galacturonic acid and 3-O-β-D-xylopyranosyl-D-galacturonic acid, by enzymolysis of lemon peel pectin (49), whereas the latter oligosaccharide has been isolated from the acidic polysaccharide of soy-bean cotyledon meal (47).

The third type of pectin splitting enzyme known as pectin-transeliminase (67) degrades pectin giving a product possessing a modified galacturonic acid residue with a double bond between C_4 and C_5, at the reducing end.



VIII

Unlike most of the polygalacturonases, pectintranseliminase catalyses the breakdown of pectin only and not of pectic acids. Using Bacillus polymixa enzyme, which attacks the non-reducing end of pectic acids, Nagel and Vaughn (68) and Starr and Moran (69) have isolated similar unsaturated products. They found that this enzyme was more active on pectic acid rather than on pectin and may be regarded as polygalacturonic acid-transeliminase. By the

action of this enzyme 4-O-(4-deoxy-β-L-threo-hex-4-enosyluronic acid)-D-galacturonic acid has been isolated (70).

Fractionation of pectins: The complex nature of pectins has directed the attention of various investigators to new methods of fractionation of these polysaccharides. Various methods have been tried to fractionate pectin preparations into acidic and neutral components and also into acidic components of varying acid content. Acidic polysaccharides can be precipitated as insoluble calcium or copper salts but there is the possibility of co-precipitation of some of the neutral polysaccharides. Neukom and co-workers (50) have chromatographed sugar beet pectin on diethyl-aminoethyl cellulose (phosphate form) and since all the fractions contained the residues of D-galactose and L-arabinose they deduced that these sugars are integral constituents of pectin. Pectins from apple, citrus fruits and sugar beet were similarly fractionated and in addition to similar acidic polysaccharides, a neutral component which was either an arabinogalactan or a mixture of arabinan and galactan, was isolated (71). However, this procedure cannot be conveniently employed to separate large quantities of polysaccharide because of the low capacity of ion-exchange medium and the large amounts of buffer solution needed to desorb the acidic polysaccharides.

Recently an ion-exchange resin of high capacity, diethylamino-ethyl-Sephadex A-50 (formate form) has been successfully used to chromatograph lemon peel pectin (49). This procedure gives a very good separation of, neutral polysaccharide, acidic polysaccharide

rich in neutral sugars, and the major component of pectinic acid. Because of the high capacity of ion-exchange resin and the use of formic acid solutions instead of phosphate buffers, to desorb the acidic polysaccharides from the column, this method can be used for a large scale separation of pectins. To test the homogeneity of the samples of pectic acids, moving boundary electrophoresis (20)(22)(59) and zone electrophoresis (59) have been used.

Timell and Bhattacharjee (22) have separated a galacturonan and a polysaccharide containing both galacturonic acid and neutral sugar residues from pectins present in the bark of amabilis fir, by acidifying the pectin solution and separating acid soluble and insoluble components on ultracentrifuge. Zitko and Bishop (20) have fractionated pectic acids obtained by saponification of pectins from sunflower heads, sugar beet, apple and citrus fruits, at different concentrations of sodium acetate solution to give galacturonans and acidic polysaccharides containing varying proportions of neutral sugars.

Extraction and Purification of Polysaccharides

The crops of alfalfa was cut in the beginning of October and plant material was separated into leaves and stems. Two series of separate extractions were carried out on separated leaves and stems in order to compare the polysaccharides present in the leaves and stems of alfalfa. The plant materials were first extracted with ethanol:water (4:1) to inactivate enzymes and to remove colouring matter and soluble sugars and then with cold water to remove water-soluble polysaccharides together with much protein. In order to isolate pectic material present in alfalfa the residue was extracted with a 2% solution of ethylenediaminetetra-acetic acid disodium salt, at 70°. The polysaccharide from the extract was precipitated with acetone and purified via the calcium salt. Calcium chloride solution was added to a solution of polysaccharide and the precipitated calcium pectate was removed at centrifuge and washed with water. A very small amount of polysaccharide recovered from the supernatant solution and washings was found to be qualitatively similar to the original alfalfa pectic acid. Calcium pectate was converted to ammonium pectate by heating at 90° with a 0·5% solution of ammonium ~~pectate~~ oxalate, the precipitated calcium oxalate was removed at centrifuge and ammonium pectate was precipitated with acetone (1 vol.). The polysaccharide was removed at centrifuge, washed with acetone:water (1:1) and dried by solvent exchange. The pectic material was once more purified through the calcium salt and the regenerated ammonium pectate was finally dissolved in water and freeze-dried.

In this way two samples of ammonium pectate were isolated from separated leaves and stems of alfalfa, (a) leaf ammonium pectate, and (b) stem ammonium pectate. The analysis of the two samples are given in table II, which show that both the samples have similar uronic anhydride and methoxyl content as well as specific rotations.

	$[\alpha]_D$	u.a.a. %	methoxyl content
(a) Leaf ammonium pectate	205 ± 5	73-75	2·5%
(b) Stem ammonium pectate	200 ± 5	73	2·3%

Table II

Samples of (a) and (b) were hydrolysed and the hydrolysates gave the similar paper chromatographic pattern, indicating the presence of following sugars in the approximate proportion given in parenthesis:-

galactose (+ +), glucose (tr.), arabinose (+ + +), xylose (tr.), fucose (tr.), rhamnose (+), 2-O-methylxylose (tr.), 2-O-methylfucose (tr.), acidic oligosaccharides (+ +).

Samples of leaf and stem pectic acids were examined by diethylaminoethyl cellulose chromatography (50). Both the polysaccharide preparations gave the same elution pattern on this ion-exchange resin. More than 95% of the polysaccharide was eluted in a single band with sodium hydroxide solution. Only traces of polysaccharide were eluted with 0·5 M-sodium phosphate buffer and found to contain the same constituent sugars as the original pectic acid.

Partial degradation of ammonium pectate

In order to obtain some detailed information about the fine structure of ammonium pectate, the polysaccharide was subjected to two types of partial degradations: (A) partial acid hydrolysis, and (B) partial acetolysis.

(A) Partial acid hydrolysis of leaf ammonium pectate

Leaf ammonium pectate was partially hydrolysed in N-sulphuric acid to give a mixture of mono- and oligo-saccharides, and degraded galacturonans, which contained 98% of uronic acid residues and on further hydrolysis gave galacturonic acid, galacturonobiose and galacturonotriose. The mixture of sugars was first fractionated on a charcoal column into two fractions, (a) eluted with water and containing predominantly monosaccharides, and (b) eluted with 35% ethanol and containing mainly acidic oligosaccharides. Both the fractions were further fractionated on the ion-exchange resin, diethylaminoethyl-Sephadex A-25 (formate form). Elution with water gave neutral sugars among which arabinose, galactose, rhamnose and traces of fucose, xylose, 2-O-methyfucose and 2-O-methylxylose were identified by paper chromatography. Elution with water containing increasing amounts of formic acid gave various fractions containing acidic oligosaccharides, which were further separated, as required, by paper partition chromatography.

In all, three types of acidic oligosaccharides were isolated, (a) acidic oligosaccharides containing residues of galacturonic acid only, (b) acidic oligosaccharides containing residues of galacturonic acid and rhamnose, and (c) aldobiouronic acids

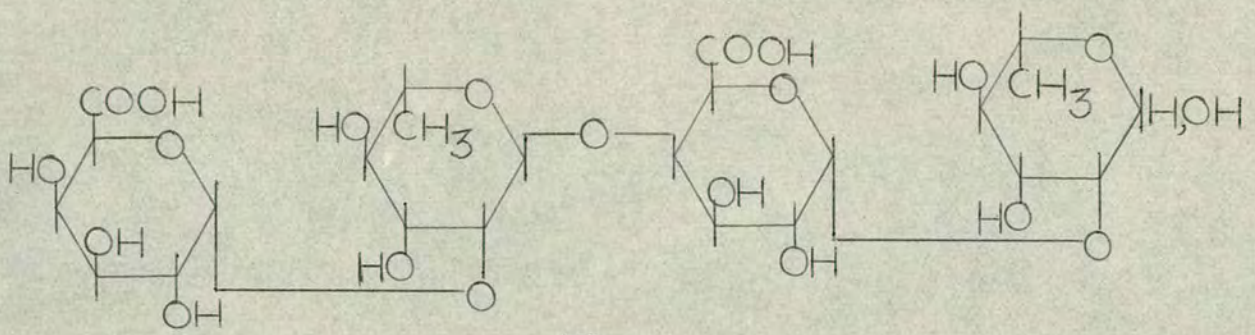
containing glucuronic acid residues as acidic sugar moiety, which were obtained in relatively small amounts.

(a) Two acidic oligosaccharides containing only galacturonic acid residues were characterised as 4-O-(α-D-galactopyranosyluronic acid)-D-galacturonic acid (Structure I, p.3) and O- -D-galacto-pyranosyluronic acid-(1 → 4)-O- α-D-galactopyranosyluronic acid-(1 → 4)-D-galacturonic acid (Structure II, p. 3) on the basis of the following observations:- The oligosaccharides were chromatographically similar to corresponding galacturonobiose and galacturonotriose obtained previously by partial hydrolysis of alfalfa (4) and other pectic acids (47)(48) and on hydrolysis gave galacturonic acid only. The methyl esters methyl glycosides of the oligosaccharides on reduction with sodium borohydride and subsequent hydrolysis gave galactose. The linkages in these oligouronides were determined by methylation analysis of the corresponding galactobiose and galactotriose obtained by reduction of galacturonobiose and galacturonotriose, respectively. Methyl ester methyl glycosides of the acidic oligosaccharides were subjected to trimethylsilylation and trimethylsilyl derivatives were reduced with lithium aluminium hydride in ethereal solution. Gas-liquid chromatography of methanolysis products from methylated carboxyl-reduced sugars gave peaks with the retention times of methyl glycosides of 2,3,4,6-tetra- and 2,3,6-tri-O-methylgalactose.

(b) Three oligosaccharides containing galacturonic acid and rhamnose residues were isolated. The first acidic oligosaccharide was chromatographically and ionophoretically similar to 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose and contained the

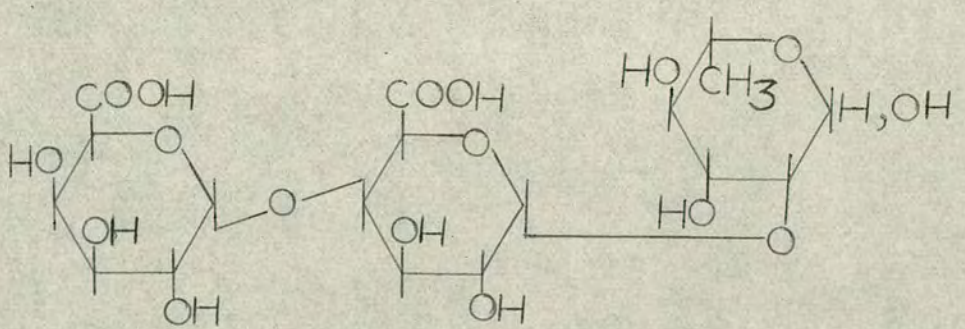
residues of galacturonic acid and rhamnose in the ratio of 1:0·93. Methanolysis of methylated aldobiouronic acid on gas-liquid chromatographic examination gave peaks with the retention times of methyl glycosides of 3,4-di-O-methylrhamnose and 2,3,4-tri-O-methyl-galacturonic acid. Structure III (p.3) for aldobiouronic acid was confirmed by the formation of crystalline methylglycoside pentamethyl ether, methyl-2-O-(2,3,4-tri-O-methyl-α-D-galacto-pyranosyluronic acid)-3,4-di-O-methyl-L-rhamnoside, dihydrate. The second oligosaccharide was chromatographically indistinguishable from the alternating tetrasaccharide, O-α-D-galactopyranosyluronic acid-(1 → 2)-O-β-L-rhamnopyranosyl-(1 → 4)-O-α-D-galactopyrano-syluronic acid-(1 → 2)-L-rhamnose (47)(48). The sugar contained galacturonic acid and rhamnose residues in the molar ratio of 2:2·17, whereas the derived glycitol contained the same sugar residues in the ratio of 2:1·2. On partial hydrolysis the sugar gave predominantly 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose with small amounts of galacturonic acid and rhamnose, while controlled partial hydrolysis of the derived glycitol gave the same aldobiouronic acid with the glycitol, 2-O-(α-D-galactopyranosyluronic acid)-rhamnitol. Gas-liquid chromatography of methanolysis products from the methylated tetrasaccharide gave peaks with the retention times of methyl glycosides of 3,4-di-O-methylrhamnose,2,3-di- and 2,3,4-tri-O-methylgalacturonic acid. The same sugars along with 1,3,4,5-tetra-O-methylrhamnitol were identified by gas chromatography, in the methanolysate of the methylated glycitol of tetrasaccharide. Assuming that both galacturonic acid residues have an α-configuration, the molecular rotation of tetrasaccharide suggests that the non-

reducing rhamnose residue has a β-configuration. These results show that the sugar is a linear molecule with the structure:



IX

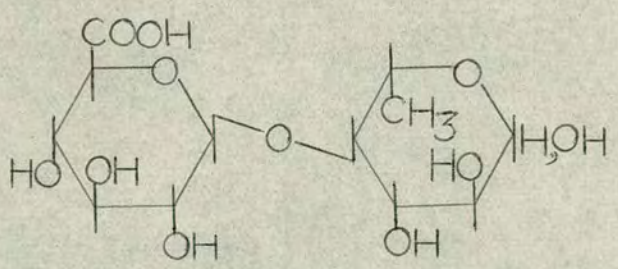
The third oligosaccharide containing galacturonic acid and rhamnose residues was chromatographically similar to O-α-D-galactopyranosyluronic acid-(1 → 4)-O-α-D-galactopyranosyluronic acid-(1 → 2)-L-rhamnose (47)(48). Hydrolysis of the sugar gave galacturonic acid, rhamnose and 2-O-(galacturonosyl)-rhamnose, whilst hydrolysis of derived glycitol gave galacturonic acid and rhamnitol. The sugar contained galacturonic acid and rhamnose residues in the molar ratio of 2:0·93. The sugar was assigned structure X on the basis that similar acidic oligosaccharide obtained by acetolysis of stem pectic acid on methylation and gas-liquid chromatographic examination of the methanolysis products from methylated sugar gave peaks with the retention times of methyl glycosides of 3,4-di-O-methylrhamnose, 2,3-di- and 2,3,4-tri-O-methylgalacturonic acid.



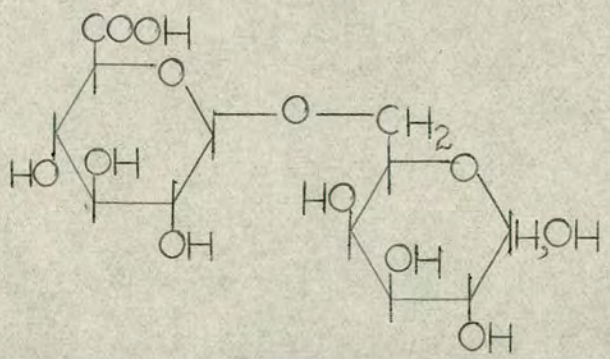
X

(c) Two aldobiouronic acid containing glucuronic acid as one of the constituent sugars were isolated in comparatively low yields. The first aldobiouronic acid was assigned structure XI on the basis of the following observations:-

The sugar was chromatographically and ionophoretically similar to 4-O-(β-D-glucopyranosyluronic acid)-L-fucose (47)(48). Hydrolysis of the sugar gave glucuronic acid, glucurone and fucose. Hydrolysis of carboxyl-reduced disaccharide obtained by sodium borohydride reduction of methyl ester methyl glycosides of aldobiouronic acid gave glucose and fucose. Gas-liquid chromatography of methanolysis products from the methylated sugar gave peaks with the retention times of methylglycosides of 2,3-di-O-methylfucose and 2,3,4-tri-O-methylglucuronic acid. The specific rotation ($[\alpha]_D$ - 72) of the aldobiouronic acid suggests a β-configuration of the linkage.



XI



XII

The second aldobiouronic acid was chromatographically and ionophoretically similar to 6-O-(β-D-glucopyranosyluronic acid)-D-galactose. Hydrolysis of the sugar gave glucuronic acid, glucurone and galactose, whereas hydrolysis of the carboxyl-reduced disaccharide gave glucose and galactose. Methanolysis of methylated aldobiouronic acid gave components with the retention

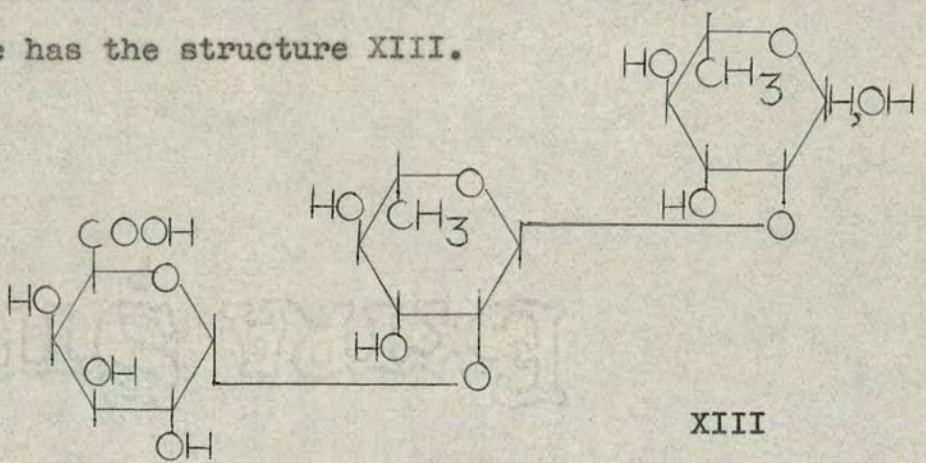
times of methylglycosides of 2,3,4-tri-O-methylglucuronic acid and 2,3,4- and 2,3,5-tri-O-methylgalactose. These results suggest structure XII for this aldobiouronic acid.

(B) Acetolysis of stem ammonium pectate:-

A sample of stem ammonium pectate was subjected to graded acetolysis, which furnished a degraded polysaccharide containing only galacturonic acid residues and a mixture of sugars containing mono- and oligo-saccharides. The sugars were fractionated on diethylaminoethyl Sephadex A-25 (formate form) ion exchange resin into a mixture of neutral sugars composed of galactose, arabinose, rhamnose, xylose, fucose, 2-O-methylfucose and 2-O-methylxylose and four fractions of acidic oligosaccharides, which were further separated, wherever necessary, by paper partition chromatography. Four acidic oligosaccharides, all containing residues of galacturonic acid and rhamnose, were obtained. The first and second oligosaccharides were characterised as 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose (III, p.3) and O-α-D-galactopyranosyluronic acid-(1 → 2)-O-(β-L-rhamnopyranosyl-(1 → 4)-O-α-D-galactopyranosyluronic acid-(1 → 2)-L-rhamnose (IX), by the same series of observations, carried out to characterise these oligosaccharides obtained by partial hydrolysis of leaf ammonium pectate.

The third oligosaccharide was chromatographically similar to the trisaccharide, O-galacturonosyl-(1 → 2)-rhamnopyranosyl-(1 → 2)-rhamnose (47)(48). The sugar contained galacturonic acid and rhamnose residues in the ratio of 1:1·97, whilst derived glycitol contained the same sugar residues in the ratio of 1:0·92. Partial

hydrolysis of trisaccharide gave 2-O-(galactopyranosyluronic acid)-rhamnose and rhamnose. Hydrolysis of the derived glycitol gave galacturonic acid, rhamnose and rhamnitol. Gas-liquid chromatography of methanolysis products from the methylated sugar gave peaks with the retention times of methyl glycosides of 3,4-di-O-methylrhamnose and 2,3,4-tri-O-methylgalacturonic acid. These results along with the specific rotation of the trisaccharide suggest that this oligosaccharide has the structure XIII.



XIII

The fourth oligosaccharide was chromatographically similar to O-(α-D-galactopyranosyluronic acid)-(1 → 4)-O-α-D-galactopyranosyluronic acid-(1 → 2)-L-rhamnose, isolated by partial hydrolysis of leaf ammonium pectate and contained the residues of galacturonic acid and rhamnose in the molar proportion of 2:0·95. Hydrolysis of the sugar gave galacturonic acid, rhamnose and 2-O-galacturonosylrhamnose, whereas hydrolysis of derived glycitol gave galacturonic acid and rhamnitol. Methanolysis of methylated oligosaccharide on gas chromatographic examination gave peaks with the retention times of 3,4-di-O-methylrhamnose and 2,3-di- and 2,3,4-tri-O-methylgalacturonic acid. These results along with the specific rotation of oligosaccharide suggest that this fragment has structure X.

Acetolysis of leaf ammonium pectate:-

As only a small amount of leaf ammonium pectate was left, the acidic oligosaccharides formed by graded acetolysis of the polysaccharide could be identified only by paper chromatography. The following three oligosaccharides, along with the same neutral monosaccharides and degraded polysaccharide, were obtained by partial acetolysis of a small sample of leaf ammonium pectate.

1. 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose.
2. O-α-D-galactopyranosyluronic acid-(1 → 2)-O-β-L-rhamnopyranosyl-(1 → 2)-L-rhamnose.
3. O-α-D-galactopyranosyluronic acid-(1 → 2)-O-β-L-rhamnopyranosyl-(1 → 4)-O-α-D-galactopyranosyluronic acid-(1 → 2)-L-rhamnose.

Methylation of carboxyl-reduced stem pectinic acid

In order to obtain more information about the linkages between the various sugars in the polysaccharide, by methylation analysis of carboxyl-reduced pectinic acid, a sample of stem pectinic acid was reduced and subsequently methylated. Reduction was carried out by the method of Rees and Samuel (72), which involves the following operations: Pectinic acid was esterified with ethylene oxide (46) to give 2-hydroxy-ethyl ester of pectinic acid, which was acetylated by the method of Carson and Maclay (73). The acetate of the 2-hydroxy-ethyl pectinic ester was reduced with lithium borohydride in tetrahydrofuran. The reduced product contained mainly the residues of galactose with traces of other neutral monosaccharides. The carboxyl-reduced polysaccharide was methylated and the methylated product methanolysed. Gas-liquid chromatographic examination of methanolysis products showed the presence of the following sugars:

Sugars	Relative amount
2,3,4-tri-O-methyl-D-xylose	+
2,3,4-tri-O-methyl-L-rhamnose	+
2,3,5-tri-O-methyl-L-arabinose	+ +
2,3,4-tri-O-methyl-L-fucose	+
3,4-di-O-methyl-L-rhamnose	+ + +
2,3-di-O-methyl-L-arabinose	+ +
2,3-di-O-methyl-L-fucose	+
3,4-di-O-methyl-D-xylose	+
2,3,4,6-tetra-O-methyl-D-galactose	+ + +
3-O-methyl-L-rhamnose	+

(contd.)

2,3,6-tri-O-methyl-D-galactose	(major component)
3,6-di-O-methyl-D-galactose	6 +
2,6-di-O-methyl-D-galactose	6 +

Evaluation of Results

The analyses of leaf and stem ammonium pectates show that the two samples of the polysaccharide are the same. Both the polysaccharide samples are also similar to the ammonium pectate extracted with ammonium oxalate in the previous investigation (4). Partial hydrolysis results of leaf ammonium pectate, discussed in the previous chapter and stem ammonium pectate (93), as well as the results of acetolysis of the two samples of the polysaccharide indicate the structural similarity in the polysaccharide components, as the same type of acidic oligosaccharides have been isolated from both the samples. It is noteworthy that the samples of xylans extracted from separated leaves and stems of alfalfa were similar in composition and structure (5). The partial depolymerisation and methylation studies on the alfalfa ammonium pectate lead to the following conclusions, regarding the structural role of various sugar residues present in the polysaccharide.

Basal chain of pectic acid:

In the previous investigation (4) the basal chain of alfalfa pectic acid was shown to consist of 1 → 4 linked α-D-galacto-pyranosyluronic acid residues by the isolation of galacturonobiose (I), galacturonotriose (II), and a degraded galacturonan, as the products of partial hydrolysis, and by methylation results of the polysaccharide. In the present investigation both of these acidic oligosaccharides have been isolated and characterised on the

basis of methylation results of the corresponding galactobiose and galactriose obtained by the carboxyl-reduction of the galacturonobiose and galacturonotriose. Partial hydrolysis of the pectic acid gave a degraded polysaccharide resistant to further hydrolysis and containing solely the galacturonic acid residues. Methanolysis of the methylated carboxyl-reduced pectic acid gave a high proportion of the methyl glycosides of 2,3,6-tri-O-methyl-D-galactose. All these results are in agreement to the previous investigation. The isolation of 2,6- and 3,6-di-O-methyl-D-galactose from the methylated carboxyl-reduced pectic acid indicates that some of the galacturonic acid units in the chain act as branch points at 3 or 2 position, although these sugars may have arisen partly due to under-methylation of the polysaccharide.

The role of L-rhamnose residues:

In the earlier work (4) L-rhamnose residues were shown to be integral constituents of the alfalfa pectic acid, by the isolation of an aldobiouronic acid, 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose (III) as a product of partial hydrolysis of the polysaccharide. During the present investigation four acidic oligosaccharides (III, IX, X and XIII) containing galacturonic acid and rhamnose residues have been isolated by partial hydrolysis and/or acetolysis of the polysaccharide. These oligosaccharides have also been isolated by partial depolymerisation of the acidic polysaccharides of soybean cotyledon meal (47) and soybean hull (48) and lemon peel pectin (49).

D-GalpA1 $\xrightarrow{\alpha}$ 2L-Rha

III

D-GalpA1 $\xrightarrow{\alpha}$ 2L-Rhap1 $\xrightarrow{\beta}$ 4D-Galp1 $\xrightarrow{\alpha}$ 2L-Rha

IX

D-GalpA1 $\xrightarrow{\alpha}$ 4D-GalpA1 $\xrightarrow{\alpha}$ 2LRha

X

D-GalpA1 $\xrightarrow{\alpha}$ 2L-Rhap1 $\xrightarrow{\beta}$ 2L-Rha

XIII

These results clearly show that in alfalfa pectinic acid, the main chain of 1 $\rightarrow$ 4 linked α -D-galactopyranosyluronic acid residues contains some 1 $\rightarrow$ 2 linked β -L-rhamnopyranose residues. The alternating tetrasaccharide IX shows an interesting structural feature of the galacturonorhamnan chain, whereas, the trisaccharide XIII indicates that at least two rhamnose residues are adjacent in parts of the structure. Alfalfa pectic acid contains a relatively small proportion of L-rhamnose residues as compared with the residues of D-galacturonic acid. Partial hydrolysis of the polysaccharide furnished a degraded galacturonan on one hand and acidic oligosaccharides such as IX and XIII on the other hand. These observations suggest that rhamnose residues are concentrated in certain parts of the chain in the structural units such as XIV, with the blocks of galacturonic acid residues.

---4D-GalpA1 $\xrightarrow{\alpha}$ 4D-GalpA1 $\xrightarrow{\alpha}$ 2L-Rhap1 $\xrightarrow{\beta}$ 4D-GalpA1 $\xrightarrow{\alpha}$ 2L-Rhap1 $\xrightarrow{\beta}$ 2L-Rhap1---

$\uparrow$3 or 2 $\qquad$ $\uparrow$4

XIV

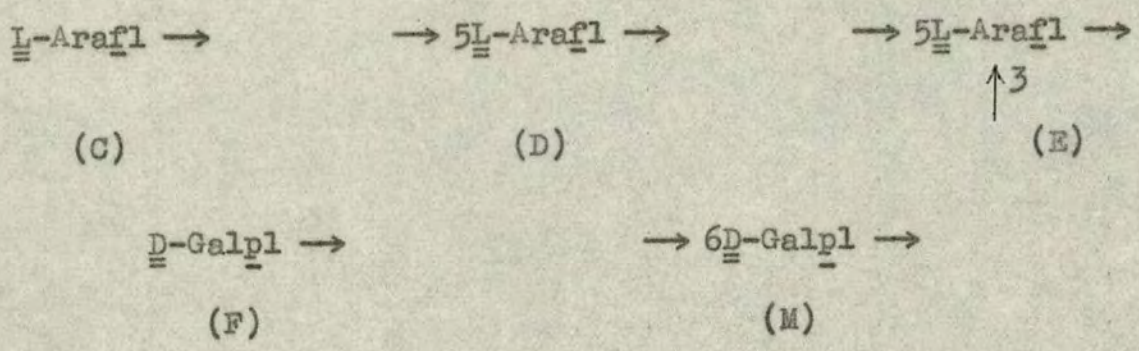
(Galacturonorhamnan basal chain)

Further information about the role of rhamnose residues in the polysaccharide is obtained by the methylation analysis of the carboxyl-reduced pectic acid. The isolation of 3,4-di-O-methyl-L-rhamnose from the methylated polysaccharide indicates the presence of 2-O- substituted L-rhamnose residues in the polysaccharide, whereas that of 2,3,4-tri-O-methyl-L-rhamnose and 3-O-methyl-L-rhamnose indicates that some of the rhamnose residues are present at the non-reducing end (former case) and certain of the rhamnose units act as branch points at position 4 (latter case) as was indicated earlier (4). The same type of galacturonorhamnan basal chains has been found to be present in the acidic polysaccharides of soybean (47)(48) and lemon peel pectin (49). The galacturonorhamnan basal chains have been found as a structural feature of a wide range of plant polysaccharides containing galacturonic acid and rhamnose residues. The polysaccharide components of exudate gums of the Sterculia (90) and Khaya (91) genera, which contain a relatively high proportion of rhamnose residues, on one hand and tragacanthic acid (96) the acidic polysaccharide fraction of gum tragacanth which contains a small proportion of rhamnose residues, on the other hand, belong to the galacturonorhamnan family.

D-Galactose and L-arabinose residues:

The methylation studies on the carboxyl-reduced alfalfa pectic acid show that L-arabinose and D-galactose residues are present in the outer chain of the polysaccharide in the structural units reported earlier (4), i.e., the galactose residues as single non-reducing end groups (F), and the arabinose residues as multiple

units (structural features C, D and E). In this respect the pectic acid resembles most closely the lemon peel pectin (49) because similar structural features have been found in the latter polysaccharide.



In contrast, the commercial citrus pectin (24) and the acidic polysaccharides of soybean (47)(48) contain the side chains of 1 → 4 linked β-D-galactopyranose residues. The conclusion was reached by the isolation of 4-O-(β-D-galactopyranosyl)-D-galactose and the higher homologues of the series obtained by the partial depolymerisation of the polysaccharides. No such oligosaccharides were obtained by the graded acetolysis of alfalfa pectic acid, which indicates the absence of multiple units of galactose residues in the pectic acid. However, a galactose containing aldobiouronic acid, 6-O-(β-D-glucopyranosyluronic acid)-D-galactose has been obtained as a product of partial hydrolysis of alfalfa pectic acid, which indicates that some of the galactose residues are present in the structural units represented by (M).

It is interesting that arabinose and galactose residues have been found to be present as single non-reducing end groups in some of the pectic materials such as the pectins from the sisal flash (46) and the bark of amabilis fir (22) on one hand, and as multiple unit

side chains on the other hand, as in the case of commercial citrus pectin (24) and the acidic polysaccharides of soybean (47)(48). The following table shows the structural features of these sugar residues in some of the pectic substances:-

Pectins from:-	Galactose		Arabinose	
	(a)	(b)	(a)	(b)
1. Alfalfa	+			+
2. Lemon peel (49)	+			+
3. Sisal flash (46)	+		+	
4. Bark of amabilis fir (22)	+		+	
5. Citrus fruits (commercial)(24)		+		+
6. Soybean cotyledon meal (47)		+		+
7. Soybean hull (48)		+		+

Table III

(a) denotes the sugar residues present predominantly as non-reducing end groups.

(b) denotes the sugar residues present in multiple units.

Arabinose and galactose residues have been found in the multiple units in some of the minor polysaccharide fractions of alfalfa. An arabinogalactan has been isolated from the polysaccharides extracted with cold water from alfalfa, which contains arabinose and galactose residues in a complex branched structure (93).

Other sugars present in the pectic acid:

Xylose, fucose, 2-O-methylxylose and 2-O-methylfucose have been identified in the stem and leaf ammonium pectates. Of these, xylose and some of the residues of fucose and/or 2-O-methylfucose

are present in the outer chain as non-reducing end groups. This is indicated by the isolation of 2,3,4-tri-O-methyl-D-xylose and 2,3,4-tri-O-methyl-L-fucose from the methylated carboxyl-reduced pectic acid. Direct evidence for the location of xylose residues in some of the pectic substances has been obtained. A pseudo aldobiouronic acid, 3-O- β-D-xylopyranosyl-D-galacturonic acid has been isolated by the enzymolysis of tragacanthic acid (95) - the acidic polysaccharide component of gum tragacanth, lemon peel pectin (49) and the acidic polysaccharide of soybean cotyledons (47), which shows that xylose residues are linked to the basal chain through the 3 position in a few of the galacturonic acid residues. Recently, the enzymolysis of alfalfa pectic acid (92) has furnished an acidic fragment containing xylose residue as non-reducing end group, which confirms that the xylose is an integral constituent of alfalfa pectic acid.

Two aldobiouronic acids containing glucuronic acid as the acidic sugar moiety, 4-O-(β-D-glucopyranosyluronic acid)-L-fucose (XI) and 6-O-(β-D-glucopyranosyluronic acid)-D-galactose (XII), have been isolated by partial hydrolysis of alfalfa leaf and stem (93) ammonium pectates. Oligosaccharide XII was also isolated by partial hydrolysis of the so-called hemicelluloses of alfalfa (3). The isolation of this oligosaccharide from the pectic material in the present investigation, confirms that the previous polysaccharide preparation (3) also contained pectic acid, which had furnished aldobiouronic acids such as 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose and 6-O-(β-D-glucopyranosyluronic acid)-D-galactose.

D-GpAl $\xrightarrow{\beta}$ 4L-Fuc

XI

D-GpAl $\xrightarrow{\beta}$ 6D-Gal

XII

The isolation of the oligosaccharides XI and XII from alfalfa pectic acid provides evidence for the presence of glucuronic acid as a constituent sugar of the pectic material. A small amount of methyl glycosides of 2,3-di-O-methyl-L-fucose is identified amongst the methanolysis products from the methylated carboxyl-reduced pectic acid, which shows the presence of 4-O-substituted residues of L-fucose in the pectic material. Nevertheless, the exact structural significance of these oligosaccharides cannot be interpreted on the basis of the present information. It is more likely that these oligosaccharides have arisen from some side chains present in the pectic acid. These aldobiouronic acids have been isolated by partial hydrolysis of lemon peel pectin (49) and the acidic polysaccharides of soybean (47)(48). Partial hydrolysis of tragacanthic acid (96) which contains glucuronic acid residues in the side chains as non-reducing end groups, has also furnished these oligosaccharides.

Sodium acetate fractionation of alfalfa and citrus pectic acids

Bishop and Zitko (20) have fractionated the pectic acids obtained from sunflower head, sugar beet, apple and citrus pectins, by graded precipitation with sodium acetate. The pectic acid gave a number of acidic polysaccharide fractions of varying uronic acid content including a galacturonan free of neutral sugars (obtained from sunflower head pectic acid only). Pectin from the bark of amabilis fir (22) has also been fractionated into two components, a galacturonan, and a pectic acid containing the galacturonic acid and neutral sugar residues.

Pectic acid obtained by saponification of alfalfa pectinic acid, which was isolated from combined leaves and stems of the plant and was similar in composition to the pectinic acids obtained from separated leaves and stems of alfalfa, was subjected to sodium acetate fractionation. Since a sufficient amount of lemon peel pectin was available, a sample of the pectic acid obtained by de-esterification of lemon peel pectin was also subjected to this type of fractionation in order to compare the results of the two fractionations.

(a) Alfalfa pectic acid:

Pectinic acid from combined leaves and stems of alfalfa, which was homogeneous by the criterion of chromatography on diethylamino-ethyl-cellulose (50) was de-esterified to give pectic acid, under the mildest possible conditions (55). The pectic acid was isolated in electrophoretically homogeneous form. Fractionation of this pectic acid by Bishop's method (20) gave four fractions precipitated at

increasing concentration of sodium acetate in the solution and a fifth fraction was obtained by precipitating the residual polysaccharide in the solution, with ethanol. Each fraction was examined for, (a) galacturonic acid content by titration, (b) specific rotation ($[\alpha]_D$) of its sodium salt, (c) possible heterogeneity by electrophoresis, and (d) the presence of neutral sugars by paper chromatography of the hydrolysate. The results are shown in table IV.

The analyses show that the polysaccharide fractions formed a series of related molecular species with no apparent discontinuities in composition. The first fraction which was precipitated at lowest sodium acetate concentration approximated most closely to galacturonan but hydrolysis showed that it still contained the various neutral sugars present in the original pectinic acid. The galacturonic acid contents of the fractions precipitated at increasing sodium acetate concentrations decreased progressively, and that of the fraction precipitated with ethanol was lower. The specific rotations were directly related to galacturonic acid content. The electrophoretic patterns gave some indication of the homogeneity of the pectic acid fractions. The original pectic acid and the various fractions had similar electrophoretic mobilities and though the peaks were not completely symmetrical, in no case was a distinct second peak observable.

Sodium acetate fractionation of alfalfa pectic acid

Pectic acid fraction No.	Conc. of sodium acetate [M]	Wt. of fraction in g.	% yield	GalA %	$[\alpha]_D$	Electrophoretic mobilities μ (x 10^5)
Pectic acid	-	-	-	81	+ 239	14·7
I	0·12	0·45	5·6	92	+ 268	15·1
II	0·14	1·1	13·8	87	+ 255	14·5
III	0·18	0·7	8·8	84	+ 248	14·9
IV	0·22	1·38	17·3	83	+ 244	14·7
V	ppted. with ethanol	2·3	34·8	78	+ 236	14·6

Table IV

(b) Fractionation of lemon peel pectic acid:

Lemon peel pectin was first fractionated by the method of Aspinall and Craig (49) on diethylaminoethyl-Sephadex. Elution with water and 0·2 M-formic acid gave two minor fractions and major pectinic acid fraction used for sodium acetate fractionation was eluted with M-formic acid. The pectinic acid was converted to pectic acid by saponification to give two fractions, (a) acid insoluble pectic acid, obtained in high yield and in electrophoretically homogeneous form, and (b) acid soluble polysaccharide

fraction rich in neutral sugars and grossly heterogeneous giving four peaks on electrophoresis.

Pectic acid was further fractionated as in the case of alfalfa pectic acid by graded precipitation with sodium acetate to give four sub-fractions and a fifth fraction was obtained by precipitating the residual polysaccharide in the solution, with ethanol. The analyses of the various fractions are given in table V.

Pectic acid fraction No.	Conc. of sodium acetate [M]	Wt. of fraction in g.	% yield	GalA %	$[\alpha]_D$	Electrophoretic mobilities μ (x 105)
Pectic acid	-	-	-	84	+ 242	13·9
I	0·12	0·7	5	94	+ 288	14·6
II	0·14	3·4	24	88	+ 265	15·1
III	0·18	5·6	40	85	+ 257	15·9
IV	0·22	1·7	12	82	+ 240	14·9
V	ppted. with ethanol	1·2	8·6	68	+ 186	15·3
Acid soluble	-	-	-	36	+ 100	15·8, 13·0 11·4, 3·1

Table V

These results show that as in the case of alfalfa pectic acid fractionation, the first fraction precipitated at lowest sodium acetate concentration was rich in galacturonic acid content but no

pure galacturonan devoid of neutral sugars was obtained. The galacturonic acid content of the fractions decreased with increasing concentration of sodium acetate and was lowest in the last fraction precipitated with ethanol. All the fractions were electrophoretically homogeneous.

The results of sodium acetate fractionation of alfalfa and lemon peel pectic acids are very different from the results of the same fractionation on the other pectic acids carried out by Zitko and Bishop (20). On the basis of sodium acetate fractionation of sunflower head, sugar beet, apple and citrus pectic acids (20), it was shown that each of the pectic acid contained two acidic components, one a galacturonan free of neutral sugars, the other a pectic acid which contained the neutral sugars as well, although the pure galacturonan was obtained only in the case of sunflower head pectic acid. The sugar beet pectic acid was supposed to be an exception as far as the presence of a galacturonan is concerned. In contrast, the fractionation of alfalfa and lemon peel pectic acids did not give the pure galacturonan devoid of neutral sugars, but furnished five acidic polysaccharide fractions, which formed a series of molecular species with no discontinuities in the composition.

To obtain further information on the structural role of the neutral sugar residues of the pectic acid, one of the pectic acid fraction (f III) was methylated. Gas-liquid chromatography of the methanolysis products from the methylated polysaccharide gave peaks with the retention times of methyl glycosides of the following sugars:

2,3,4-tri-O-methyl-D-xylose

2,3,5-tri-O-methyl-L-arabinose

2,3-di-O-methyl-L-arabinose

2,3,4-tri-O-methyl-L-fucose

2,3-di-O-methyl-L-fucose

2,3,4-tri-O-methyl-L-rhamnose

3,4-di-O-methyl-L-rhamnose

3-O-methyl-L-rhamnose

2,3,4,6-tetra-O-methyl-D-galactose

2,3,6-tri-O-methyl-D-galactose

2,3-di-O-methyl-D-galacturonic acid

2,3,4-tri-O-methyl-D-galacturonic acid

These results indicate that the residues of various neutral sugars, i.e., xylose, arabinose, fucose, rhamnose (in part) and galactose are present in the outer chain of the polysaccharide. The methyl ethers of D-galacturonic acid and L-rhamnose residues indicate the presence of galacturonorhamnan chain in the polysaccharide and that some of the rhamnose units act as branch points at position 4. The polysaccharide fraction resembles very closely the original pectic acid. In the previous structural investigation of lemon peel pectin (49), the galacturonorhamnan basal chain was shown to contain galactose and xylose residues predominantly as single unit side chains and arabinose residues in a branched arabinan type of structure.

The isolation of 2,3-di-O-methyl-L-fucose from the methylated polysaccharide shows the presence of some 4-O-substituted residues of L-fucose in the polysaccharide, which is in good agreement with the partial hydrolysis results of the citrus pectin, which has furnished an aldobiouronic acid, 4-O-(β -D-glucopyranosyluronic

acid)-L-fucose (XI) as one of the products.

The results of methylation of this pectic acid fraction also resemble the methylation results of (a) carboxyl-reduced alfalfa pectic acid, discussed previously and (b) acidic polysaccharide of soybean cotyledon meal (94).

EXPERIMENTAL

General methods

Paper partition chromatography:-

Paper chromatography was carried out on Whatman No.1 and No.4 filter papers. The following solvent systems (v/v) were employed to develop the chromatograms:-

(A) ethyl acetate-pyridine-water (10:4:3)

(B) ethyl acetate-acetic acid-formic acid-water (18:3:1:4)

(C) ethyl acetate-acetic acid-formic acid-water (18:8:3:9)

(D) ethyl acetate-pyridine-acetic acid-water (5:5:1:3)

(E) methyl ethyl ketone-acetic acid-water (9:1:1, saturated with boric acid)

(F) butanol-ethanol-water (4:1:5; upper layer)

(G) butanol-acetic acid-water (4:1:5; upper layer).

Detection of sugars on chromatograms:-

(A) Reducing sugars were detected by spraying the dried chromatograms with a solution of either aniline oxalate in ethanol or p-anisidine in butan-1-ol.

(B) Sugar alcohols were detected with the periodate-permanganate spray reagent (74).

(C) Polyhydroxy compounds in general, whether reducing or not, were detected with alkaline silver nitrate (75). The spray was particularly useful for the detection of small quantities.

The following abbreviations have been used to describe the chromatographic mobilities of sugars:-

R_f is equal to the ratio of the distance travelled by the component to that travelled by solvent.

R_{GalA} refers to the distance travelled by the sugar compared with that of D-galacturonic acid.

Preparative paper chromatographic separations were carried out on Whatman 3 MM and 3 MC filter sheets, the papers having been extracted overnight with water in a Soxhlet extractor. The positions of the components were located by cutting off narrow strips, which were developed with spray reagent A. The appropriate parts of the filter sheets were then cut out and the sugar residues eluted with water.

Paper ionophoresis:-

Ionophoresis (76) was carried out on Whatman No.1 paper in barate buffer (pH, 10) at a potential of 350 volts, for 4-6 hours. Spraying was carried out with the usual aniline oxalate spray to which 10% glacial acetic acid had been added. The M_G value of sugar refers to its mobility relative to D-glucose.

Moving boundary electrophoresis (59) of polysaccharides was carried out in pyridine-acetic acid buffer solution (1% pyridine and 0·4% acetic acid giving a pH 4·5) using a Tiselius-type, Spinco Model H apparatus (The experiments were performed by Mr. I.W. Cottrell).

Column chromatography:-

Charcoal columns were packed as a slurry of charcoal. The activated charcoal was prewashed by decantation with three portions of boiling water. The column was thoroughly washed with water before applying the sugar mixture.

DEAE-Cellulose columns (50) were prepared as follows. The powder was washed alternatively with 0·5 N-hydrochloric acid and 0·5 N-sodium hydroxide solution, twice. The cellulose was washed free from alkali with water and packed as a slurry. Diethylaminoethyl-cellulose was then generated in the phosphate form by elution with 0·5 M-sodium dihydrogen phosphate buffer (pH, 6). The support was then equilibrated with 0·005 M-sodium dihydrogen phosphate buffer (pH, 6).

DEAE-Sephadex columns were packed as a slurry with the diethyl-aminoethyl-Sephadex in the formate form. The powder was first allowed to swell in water. It was then washed with 0·5 N-hydrochloric-acid, followed by distilled water until free from chloride ions and then with 0·5 N-sodium hydroxide solution followed by distilled water until free from base. The procedure was repeated with further portions of acid and alkali. Finally, diethylaminoethyl-Sephadex was generated in the formate form by stirring three times with 15% formic acid for 15 minutes. The column was then packed and free acid was removed by washing with water, until the eluate had a pH 4. DEAE-Sephadex A-25 was used to fractionate mixtures of mono- and oligo-saccharides, while A-50 (49) was used to fractionate the polysaccharides.

All evaporations were carried out under reduced pressure at or below 40°. Special care was taken to concentrate the volatile methylglycosides of some methylated sugars (77).

Small scale hydrolyses were carried out by heating the material (2-10 mg.) in N-sulphuric acid (0·5 - 2·0 ml.) in a sealed tube at 100°, for periods varying from eight (for oligosaccharides) to eighteen hours (in the case of polysaccharides). The solutions were neutralized with barium carbonate and filtered. After removing barium ions with Amberlite resin IR-120(H), the solutions were concentrated and examined by paper chromatography. Samples of oligosaccharides were partially hydrolysed by heating with 0·1 - 0·2 N-sulphuric acid (1 ml.) at 100° for 4-6 hours. Cations were removed from sugar solutions with Amberlite resin IR-120 (H) and anions with IR-45 (OH).

Optical rotations were measured at 18 ± 2° in aqueous solutions unless otherwise stated.

Borohydride reductions (78) were carried out by the addition of sodium borohydride to an aqueous solution of sugar and allowing the solution to stand overnight. Excess of borohydride was destroyed with Amberlite resin IR-120 (H). The solution was evaporated to dryness and the boric acid was removed by exhaustive evaporation (5-6 times) with methanol.

Methoxyl contents were estimated by the semimicro Ziesel method (79).

Uronic acid anhydride contents (u.a.a.) were determined by:-

(a) Decarboxylation method (80).

(b) Carbazole colorimetric method (81).

(c) Potentiometric titration method (20).

Total sugar contents of solutions were determined using the phenol sulphuric acid colorimetric method (82). Reference sugars used were L-arabinose or D-galacturonic acid.

L-Rhamnose residues were estimated by L-cystein method (83).

Small scale methylations were carried out by Kuhn procedure (84). Oligosaccharide (0·5 - 2 mg.) was dissolved in N, N-dimethyl-formamide (0·5 ml.) and methyl iodide (1 ml.) and silver oxide (0·2 g.) were added. The mixture was shaken for 24 hours at room temperature in the dark. Excess chloroform was added and precipitate removed at centrifuge. The chloroform solution was extracted with water to remove N, N-dimethylformamide, dried over sodium sulphate and concentrated to dryness.

Methyl glycosides and methyl ester methyl glycosides were prepared by treating the sugar at 100° in a sealed tube with methanolic 4% hydrogen chloride. The solution was neutralized with silver carbonate, filtered and evaporated to dryness. Four hours were sufficient for methylated neutral oligosaccharides, but longer reaction times (up to eighteen hours) were allowed for the

methanolysis of methylated acidic oligosaccharides and polysaccharides.

Gas-liquid partition chromatography (85)(86) of methyl glycosides of methylated sugars was carried out on a "Pye Argon Chromatograph" using the following columns:-

(a) 3% or 5% by weight of neopentylglycol adipate polyester on dichloro-dimethylsilane treated Celite (80-100 mesh.)

(b) 15% by weight of butan-1,4-diol succinate polyester on dichloro-dimethylsilane treated Celite.

(c) 15% by weight of polyethylene glycol adipate on dichloro-dimethylsilane treated Celite.

(d) 10% by weight of polyphenylether [m-bis(m-phenoxy-phenoxy) benzene] on dichloro-dimethylsilane treated Celite.

Operating temperatures used were, 125°, 150° and 175° for (a), 175° for (b), 150° and 175° for (c) and 200° for (d).

The retention times (T) of methyl ethers methyl glycosides are relative to that of methyl-2,3,4,6-tetra-O-methyl-β-D-gluco-pyranoside.

Organic Solvents were purified and dried by methods quoted by Vogel (87).

Extraction and purification of polysaccharides from alfalfa

Freshly cut alfalfa was separated into leaves and stems and two series of extractions were carried out on separated leaves and stems. A typical example of extraction of stems is described here. The polysaccharides from leaves and more materials from stems were extracted following the same operations.

Alcohol extraction:- The stems were chopped into 1-2" pieces and extracted with 80% ethanol in a Soxhlet apparatus, to inactivate enzymes and to remove colouring matter and soluble sugars. The dried hay was milled and once again extracted with 80% ethanol.

Cold water extraction:- Dried material (390 g.) was extracted with cold water (3 x 10 l.) for twenty-four hours. The water soluble polysaccharides (6 g.) were precipitated after concentration of the extract, with ethanol (1 vol.).

EDTA extraction:- Cold water extracted material was extracted with a 2% solution of ethylenediaminetetra-acetic acid disodium salt (3 x 3 l.) at 70° for 3 hours. After cooling the mixture, the solution was filtered through glass fibre paper and dialysed against running water. The polysaccharide was then precipitated with ethanol (1 vol.), removed at centrifuge and washed a few times with ethanol:water (1:1).

Purification of pectic material through the calcium salt:-

The polysaccharide was dissolved in water and 10% calcium chloride solution was added till the complete precipitation of the polysaccharide as calcium pectate, which was filtered off and washed with water. The filtrate was dialysed, concentrated and poured into acetone. The precipitated polysaccharide (0·25 g.) was dried by solvent exchange and found to be qualitatively similar to alfalfa pectic acid (4). Calcium pectate was suspended in a solution of ammonium oxalate (0·5%; 5 l.) and heated at 90° for 30 minutes. Precipitated calcium oxalate was removed at the centrifuge, ethanol (1 vol.) was added to the supernatant solution and the precipitated ammonium pectate was washed with ethanol-water (1:1) a few times and then with ethanol. Ammonium pectate was again precipitated as calcium pectate and the regenerated ammonium pectate (12 g.) was precipitated with ethanol, finally dissolved in water and freeze-dried.

Analysis of leaf and stem ammonium pectates:-

	$[\alpha]_D$	u.a.a.* %	methoxyl content
I Stem ammonium pectate	200 ± 5	73	2·3%
II Leaf ammonium pectate	205 ± 5	73-75	2·5%

* the carbazole method.

(Table I)

Hydrolysis of both the samples in N-sulphuric acid and paper chromatographic examination of hydrolysates in solvents (A), (B) and (F), indicated the presence of galactose, arabinose, rhamnose,

small amounts of glucose, xylose, fucose, 2-O-methyfucose and 2-O-methylxylose together with galacturonic acid and a complex mixture of acidic oligosaccharides.

DEAE-cellulose chromatography (50) of leaf and stem ammonium pectates:-

Leaf ammonium pectate (200 mg.) was adsorbed on a diethylaminoethyl cellulose (phosphate form) column (30 g.; 2·5 x 20 cm.). The column was eluted in a stepwise manner with sodium dihydrogen phosphate buffers at pH 6, of the following molarities:-

(a) 0·05 M (500 ml.) (b) 0·10 M (500 ml.)

(c) 0·25 M (500 ml.) (d) 0·50 M (500 ml.)

Elution was continued with a gradient of sodium hydroxide solution (0·00 - 0·30 M). Fractions (20 ml.) were collected every hour. The amount of polysaccharide present per fraction was estimated by the phenol-sulphuric acid colorimetric method (82) and the uronic acid by the carbazole method (81). A plot of polysaccharide content per fraction against tube number indicated that more than 95% of the material was eluted in a single band with sodium hydroxide solution. A small amount (c.a., 2%) of the polysaccharide eluted with 0·5 M-sodium dihydrogen phosphate solution, on hydrolysis gave the same constituent sugars as the original polysaccharide.

Stem ammonium pectate on diethylaminoethyl-cellulose column chromatographic examination gave the same elution pattern.

Partial acid hydrolysis of leaf ammonium pectate

Ammonium pectate (15 g.) was hydrolysed in N-sulphuric acid (500 ml.) on a boiling water bath for six hours. After cooling the solution, degraded polysaccharide was precipitated with acetone (1 vol.), removed at centrifuge and washed with acetone-water (1:1) and acetone. The supernatant solution and washings were concentrated to remove acetone, neutralized partly with saturated solution of barium hydroxide and finally with barium carbonate. The barium salts were removed at the centrifuge and washed with water. The supernatant solution and washings were concentrated to a reasonable volume, passed through Amberlite resin IR-120 (H) to remove barium ions and concentrated to a syrup (4·68 g.)

The degraded polysaccharide was rehydrolysed under the same conditions to give two samples of degraded polysaccharides, (a) acid soluble degraded polysaccharide (1·2 g.) and (b) acid insoluble degraded polysaccharide (7·05 g.) [found: uronic anhydride (potentiometric titration method), 98%], and a mixture of sugars (0·21 g.). Paper chromatographic examination of mixture of sugars showed the presence of galacturonic acid, galacturonobiose and galacturonotriose.

The mixture of sugars from the first hydrolysis was adsorbed on a charcoal column (200 g.; 4 x 50 cm.) and allowed to stand overnight. The column was eluted with water (3·5 l.) at a rate of 40 ml. per hour to give fraction A-1 (3·35 g.) which contained predominantly monosaccharides with small amounts of oligosaccharides. The column was then eluted with 35% ethanol (4 l.) to give fraction A-2 (1·2 g.), which was a complex mixture of acidic oligosaccharides with small amounts of neutral sugars. Each fraction was further

fractionated on diethylaminoethyl Sephadex A-25.

Fractionation of A-2 on DEAE-Sephadex A-25

The mixture of sugars was adsorbed on a column of diethylaminoethyl Sephadex (15 g.; 15 x 3 cm.; formate form). Neutral sugars (0·338 g.) were eluted with water (500 ml.) until the eluant gave a negative reaction with phenol-sulphuric acid colorimetric test. Paper chromatography of the mixture in solvent (A), (F) and (G) showed the presence of galactose, arabinose, fucose, rhamnose, 2-O-methylfucose and 2-O-methylxylose.

The acidic sugars were desorbed from the column by elution with water containing 0 → 2% formic acid, followed by 2% and 5% formic acid solutions. Fractions (12 ml.) were collected and the contents of every 10th test tube and also of some intermediate test tubes, wherever necessary, were concentrated and examined by paper chromatography in solvent system (B). Similar fractions were combined, concentrated to a small volume, formic acid was extracted with ether, and finally the mixtures of sugars were concentrated to syrups. The following fractions were obtained:-

Eluant	Fraction No.	Wt. in g.	R_{GalA} (Solvent B)	Stain with aniline oxalate
water ↓ 2% formic acid	I	0·115	0·2	brown
			0·45	yellow
			0·6 (faint)	yellow
			0·8 + traces of neutral sugars	orange
	II	0·174	0·09	orange
			0·3 (faint)	red
			0·45	yellow
			0·8	orange
			1·0	brown
	III	0·162	0·1	orange
			0·45 (faint)	yellow
			0·8	orange
			1·0	brown
	IV	0·042	0·1	orange
			0·2	brown
			0·8 (faint)	orange
			1·0	brown
2% formic acid	V	0·094	0·07	brown
			0·2	brown
			1·0	brown
5% formic acid	VI	0·074	Immobile	-
			0·2 (faint)	brown
			1·0	brown

Table II

Fractionation of A-1 on DEAE Sephadex A-25

Fraction A-1 was fractionated into a mixture of neutral sugars (1·85 g.) and five subfractions, containing acidic sugars, shown in table III, following the same procedure as in previous fractionation.

The mixture of neutral sugars on paper chromatographic examination in solvents (A), (F) and (G) showed the presence of galactose, glucose, arabinose, xylose, fucose and rhamnose.

Eluant	Fraction No.	Wt. in g.	R_{GalA} (Solvent B)	Stain with aniline oxalate
water ↓	I	0·034	0·1 (faint)	-
			0·3	red
			0·6	yellow
			0·75	orange
	II	0·058	0·1	orange
			0·24	brown
			0·75	orange
			1·0 (faint)	-
2% formic acid	III	0·633	0·1	-
			0·24	brown
			0·75 (faint)	-
			1·0 (major)	brown
2% formic acid	IV	0·505	0·06	brown
			0·1	-
			0·24	brown
			1·0 (major)	brown
5% formic acid	V	0·09	0·06	brown
			0·2	brown
			1·0	brown
			+ Immobile	

Table III

The individual acidic oligosaccharides were separated by paper partition chromatography in solvents (B) or (C). The following acidic oligosaccharides were obtained:-

Oligosaccharide 1 (170 mg.) R_{GalA} = 0·80 (solvent B), M_G = 0·55

$[\alpha]_D$ = +90 (c 0·6)

The sugar gave galacturonic acid and rhamnose on hydrolysis and was chromatographically and ionophoretically indistinguishable from 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose. The amount of galacturonic acid and rhamnose in the oligosaccharide was estimated by the carbazole (81) and L-cystein (83) methods, respectively and found to be 1:0·93. The oligosaccharide (2-4 mg.) was methylated by Kuhn's method (84). The resulting methylated product was methanolysed and the products of methanolysis were examined by gas-liquid chromatography. Peaks were obtained with the retention times of the methyl glycosides of the following sugars.

Sugars	T column (a)	T column (b)
3,4-di-O-methyl-L-rhamnose	0·85	0·98
2,3,4-tri-O-methyl-D-galacturonic acid*	6·88	7·00

* as methyl ester

Table IV

Preparation of crystalline methylated derivative by Haworth's method (88)

The sugar (100 mg.) was dissolved in water (1 ml.) and methyl sulphate (1 ml.) and sodium hydroxide solution (2 ml.; 30%) were added dropwise over a period of six hours with continuous stirring. The reaction flask was surrounded by an ice bath and an atmosphere of nitrogen was maintained throughout the reaction. Four more additions of reagents were made on consecutive days. Twenty-four hours after the final addition had been made, the solution was heated at 100° for one hour, allowed to cool, the pH was adjusted to 4 with dilute sulphuric acid solution and sodium sulphate was precipitated by the addition of methylated spirits (8 vol.). The precipitate was removed at the centrifuge and washed with methylated spirits. The supernatant liquid and washings were combined, brought to pH 8 and concentrated to remove methylated spirits. The solution was again acidified (pH 4) and extracted with chloroform (6 x 50 ml.). The chloroform extract after drying over sodium sulphate, was concentrated. The methylated disaccharide, 2-O-(2,3,4-tri-O-methyl-α-D-galactopyranosyluronic acid)-3,4-di-O-methyl-L-rhamnose dihydrate was crystallised from a mixture of chloroform-light petroleum (b.p. 100/120) and had m.p. and mixed m.p. 68°, $[\alpha]_D^{in}$ = +98 (c, 1·0/chloroform) and gave an X-ray powder photograph identical to that of an authentic sample.

Oligosaccharide 2 (20 mg.) R_{GalA} = 0·45 (solvent B), M_G = 0·63, $[\alpha]_D$ = -72 (c 0·33).

The oligosaccharide was chromatographically and electrophoretically similar to 4-O-(β-D-glucopyranosyluronic acid)-L-fucose. Hydrolysis of the sugar and examination of the products in solvents (A), (B) and (D), gave glucuronic acid, glucurone and fucose. Methyl ester methyl glycosides of the sugar were reduced with sodium borohydride. The reduced product on hydrolysis gave glucose and fucose. Gas-liquid chromatography of the methanolysis products from the methylated sugar (Kuhn's method) gave peaks with the retention times of the methyl glycosides of the following sugars:-

Sugars	T column (b)	T column (d)
2,3-di-O-methyl-L-fucose	1·06, 1·52, 2·07	0·61, 0·88, 0·97
2,3,4-tri-O-methyl-D-glucuronic-acid*	2·33, 3·16	1·8, 2·24

* as methyl ester

Table V

Oligosaccharide 3 (12 mg.) R_{GalA} = 0·18 (solvent B), M_G = 1·0

The sugar was chromatographically and ionophoretically similar to 6-O-(β-D-glucopyranosyluronic acid)-D-galactose. Hydrolysis of the sugar and chromatographic examination of the hydrolysate in solvent systems (A), (B) and (D) showed the presence of glucuronic acid, glucurone and galactose. Methyl ester methyl glycosides of the aldobiouronic acid were reduced with sodium borohydride. The

reduced product on hydrolysis gave galactose and glucose. The disaccharide was methylated by Kuhn's method and the methylated product methanolysed. Gas-liquid chromatography of the methanolysate gave peaks with the retention times of the methyl glycosides of the following sugars:-

Sugars	T column (a)
2,3,4-tri-O-methylglucuronic acid*	2·36, 3·11
2,3,5-tri-O-methyl-D-galactose	4·05
2,3,4-tri-O-methyl-D-galactose	6·88

* as methyl ester

Table VI

Oligosaccharide 4 (65 mg.) R_{GalA} = 0·1 (solvent B), 0·55 (solvent C) $[\alpha]_D$ = +92 (c 0·5)

The sugar was chromatographically similar to O-(α-D-galactopyranosyluronic acid)-(1 → 2)-O-β-L-rhamnopyranosyl-(1 → 4)-O-(α-D-galactopyranosyluronic acid)-(1 → 2)-L-rhamnose. Hydrolysis of the sugar gave galacturonic acid, rhamnose and oligosaccharide 1. Partial hydrolysis of the derived glycitol and paper chromatographic examination of the products in solvent system (E) showed the presence of oligosaccharide 1 and the glycitol, 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnitol. The sugar contained galacturonic acid and rhamnose residues (colorimetric estimation by the carbazole (81) and L-cystein (83) methods) in the molar ratio of 2:2·17, whereas the

derived glycitol contained the same residues in the ratio of 2:1·2. Gas-liquid chromatography of the methanolysis products from the methylated tetrasaccharide (Kuhn's method) gave peaks with the retention times of the methyl glycosides of the following sugars:-

Sugars	T Column (b)
3,4-di-O-methyl-L-rhamnose	0·97
2,3,4-tri-O-methyl-D-galacturonic acid*	7·01
2,3-di-O-methyl-D-galacturonic acid*	5·2

* as methyl ester

Table VII

The derived glycitol of the tetrasaccharide was also methylated by Kuhn's method (84). Gas-liquid chromatography of methanolysis products from the methylated glycitol of tetrasaccharide gave peaks with the retention times of 1,3,4,5-tetra-O-methylrhamnitol [T, 1·07, column (b)] and the methyl glycosides of the following sugars:-

Sugars	T column (b)
3,4-di-O-methyl-L-rhamnose	0·98
2,3-di-O-methyl-D-galacturonic acid*	5·2
2,3,4-tri-O-methyl-D-galacturonic acid*	6·72

* as methyl ester

Table VIII

Oligosaccharide 5 (11 mg.) R_{GalA} = 0·4 (solvent C); 0·1 (solvent B)
$[\alpha]_D$ = +82 (c 0·44)

The sugar was chromatographically indistinguishable from O-(α-D-galactopyranosyluronic acid)-(1 → 4)-O-(α-D-galactopyranosyluronic acid)-(1 → 2)-L-rhamnose. Hydrolysis of the sugar gave galacturonic acid and rhamnose, whereas the hydrolysis of derived glycitol gave galacturonic acid and rhamnitol. The sugar contained galacturonic acid and rhamnose residues in the molar ratio of 2:0·93, estimated by the carbazole (81) and L-cystein (83) methods, respectively.

Oligosaccharide 6 (55 mg.) R_{GalA} = 0·2 (solvent B) and 0·5 (solvent C)

Hydrolysis of the sugar gave galacturonic acid only. The sugar was chromatographically similar to 4-O-(α-D-galactopyranosyluronic acid)-D-galacturonic acid. The methyl ester methyl glycosides of the sugar were reduced with sodium borohydride. Hydrolysis of the reduced disaccharide gave galactose only. The percentage of galacturonic acid in the derived glycitol was estimated by the carbazole colorimetric method (81) and found out to be 59%.

Reduction and subsequent methylation of disaccharide

The sugar (35 mg.) was treated overnight with methanolic 1% hydrogen chloride (5 ml.). The solution was neutralized with silver carbonate and concentrated to a syrup which was dried over phosphorus pentoxide in vacuum for 24 hours. The dried product was dissolved in pyridine (3 ml.) and a mixture (1 ml.) of hexamethyldisilazane and chlorotrimethylsilane (2:1) was added and the mixture was shaken

vigorously for 30 seconds. After standing at room temperature for one hour, the solution was evaporated to dryness and the residue was extracted with ether (3 x 20 ml.). The combined ether extracts were evaporated to dryness to give a syrup (38 mg.), which was re-dissolved in ether (5 ml.) and refluxed with lithium aluminium hydride (100 mg.) for two hours, with vigorous stirring. Ethyl acetate was then added dropwise to the cooled solution to destroy the remaining lithium aluminium hydride, till the evolution of hydrogen ceased. The organic solvents were evaporated under reduced pressure and the residue dissolved in 0·5 N-sulphuric acid. The solution was neutralized with barium carbonate and centrifuged off. Barium ions were removed with Amberlite resin IR-120 (H) and the deionised solution was concentrated to a syrup (18 mg.). A small sample of the product was hydrolysed and gave only galactose.

The reduced product was methylated by Haworth's method (88) following the same procedure as described in the methylation of oligosaccharide 1. The methylated disaccharide was methanolysed and the products were examined by gas-liquid partition chromatography. Peaks with the retention times of the methyl glycosides of the following sugars were obtained:-

Sugars	T column (c)
2,3,4,6-tetra-O-methyl-D-galactose	1·80
2,3,6-tri-O-methyl-D-galactose	3·05, 4·10, 4·51

Table IX

Oligosaccharide 7 (30 mg.) R_{GalA} = 0·05 (solvent B), 0·2 (solvent C).

The sugar was chromatographically similar to O-(α-D-galactopyranosyluronic acid)-(1 → 4)-O-(α-D-galactopyranosyluronic acid)-(1 → 4)-D-galacturonic acid, and on hydrolysis gave galacturonic acid only. The methyl ester methyl glycosides of the sugar were reduced with sodium borohydride and the hydrolysis of reduced product gave galactose.

The sugar (20 mg.) was treated with methanolic 1% hydrogen chloride to give methyl ester methyl glycosides, which were dissolved in pyridine (2 ml.) and treated with a mixture (0·7 ml.) of hexamethyldisilazane and chlorotrimethylsilane (2:1). The trimethylsilyl derivative thus formed was reduced with lithium aluminium hydride, in ethereal solution, as described previously. The carboxyl-reduced trisaccharide was methylated by Haworth's method (88). Methanolysis of the methylated reduced trisaccharide and examination of the products by gas chromatography gave peaks with the retention times of methyl glycosides of the following sugars:-

Sugars	T Column (c)
2,3,4,6-tetra-O-methylgalactose	1·80
2,3,6-tri-O-methylgalactose	3·07,4·15,4·50

Table X

Acetolysis of stem and leaf ammonium pectates

(a) Acetolysis of stem ammonium pectate

1. Acetylation (73):-

Stem pectinic acid (8 g.) was dispersed in formamide (200 ml.) by vigorous stirring for 24 hours at room temperature. The temperature was then raised to 40° and pyridine (50 ml.) (freshly distilled over potassium hydroxide) was added dropwise with continuous stirring. After stirring for another hour acetic anhydride (150 ml.) and a fresh batch of pyridine (170 ml.) were added simultaneously over a period of six hours. The acetylation was completed by stirring the mixture overnight. The solution was then poured into ice-cold hydrochloric acid solution (2 $\underline{N}$; 1·5 l.). The precipitated polysaccharide acetate was removed at the centrifuge and washed with water. The acetate was dissolved in acetone and water removed by azeotropic distillation with chloroform. By this process the acetate was eventually chloroform soluble. The dried product was finally dissolved in chloroform (50 ml.) and precipitated by pouring into light petroleum (b.p. 60-80). After washing with light petroleum, the polysaccharide acetate (8·0 g.) was dried over paraffin wax.

2. Acetolysis of polysaccharide acetate

The polysaccharide acetate (8·0 g.) was dissolved in acetic acid (50 ml.) and acetic anhydride (50 ml.), and concentrated sulphuric acid (5 ml.) was added dropwise with vigorous stirring

at 0° over two hours. After stirring for a further three hours, the mixture was kept at room temperature for six days. The mixture was then poured into ice-water and centrifuged. The residue was extracted with chloroform. The supernatant solution was brought to pH 3 with sodium bicarbonate and then extracted with chloroform (4 x 250 ml.). The combined chloroform extracts were dried over sodium sulphate and concentrated to a syrup. The syrup was dissolved in dry methanol (25 ml.) and deacetylated with methanolic barium methoxide (0·5 N; 100 ml.). After leaving at 0° for twenty-four hours, the solution was poured into water, neutralized with dilute sulphuric acid and filtered. After removing the barium ions with Amberlite resin IR-120 (H), the solution was concentrated to a syrup (0·88 g.). Paper chromatography of the syrup in solvents (A) and (B) showed it to be a complex mixture of mono- and oligo-saccharides.

Fractionation of mixture of sugars on DEAE-Sephadex A-25

The mixture of sugars was fractionated on diethylaminoethyl-Sephadex column (15 g.; 15 x 2 cm.; formate form). Neutral sugars (0·336 g.) were eluted with water (2 l.) and the paper chromatography of the mixture showed the presence of galactose, arabinose, rhamnose, small amounts of glucose, xylose, fucose, 2-O-methylfucose and 2-O-methylxylose. Acidic sugars were then eluted from the column, successively with 0·05 M-formic acid, a gradient of 0·05 M → 0·4 M-formic acid, 0·4 M and 0·5 M- formic acid. The following fractions were obtained:-

Eluant	Fraction No.	Wt. in mg.	R_{GalA} of Spots
0·05 M- formic acid	I	42	0·4 (major), (solvent B)
	II	37	0·4, 0·8 (major), (solvent B)
	III	50	0·8, 1·0 (major), (solvent B)
0·05 M → 0·4 M-formic acid	IV	61	0·55, 1·0, (solvent C)
0·4 M-formic acid	V	37·5	0·4 (major), (solvent C)

Table XI

The individual acidic oligosaccharides were separated by filter sheet chromatography in solvent (B) or (C). The following oligosaccharides were obtained:-

Oligosaccharide 1 (17 mg.) R_{GalA} = 0·8 (solvent B)

$[\alpha]_D$ = +90 (c, 0·6)

On hydrolysis the sugar gave galacturonic acid and rhamnose and the colorimetric estimation (81)(83) of the two component sugars in the aldobiouronic acid, showed the proportion as 1:1·06. Gas-liquid chromatography of the methanolysis products from the methylated sugar gave peaks with the retention times of the methyl glycosides of 3,4-di-O-methyl-L-rhamnose and 2,3,4-tri-O-methyl-D-galacturonic acid.

Oligosaccharide 2 (15 mg.) R_{GalA} = 0·4 (solvent B), $[\alpha]_D$ = +60° (c, 0·5)

Hydrolysis of the sugar gave galacturonic acid, rhamnose and oligosaccharide 1, while partial hydrolysis gave rhamnose and oligosaccharide 1. Hydrolysis of the derived glycitol gave galacturonic acid, rhamnose and rhamnitol. The sugar contained galacturonic acid and rhamnose residues (carbazole (81) and L-cystein (83) methods) in the molar ratio of 1:1·87, while the derived glycitol contained the same residues in the ratio of 1:0·92. The methanolysis products from the methylated sugar, on examination by gas-liquid chromatography gave peaks with the retention times of the methyl glycosides of the following sugars:-

Sugars	T column (b)	T column (a)
3,4-di-O-methyl-L-rhamnose	1·00	0·85
2,3,4-tri-O-methyl-D-galacturonic acid*	6·95	6·68

* as methyl ester

Table XII

Oligosaccharide 3 (15 mg.) R_{GalA} = 0·55 (solvent C), 0·1 (solvent B) $[\alpha]_D$ = +92 (c, 0·5).

The sugar was chromatographically indistinguishable from the previously characterised tetrasaccharide (oligosaccharide 4 from partial hydrolysis) and contained galacturonic acid and rhamnose residues in the ratio of 2:1·90, while the derived glycitol contained the same residues in the ratio of 2:1·06. On

partial hydrolysis, the sugar gave oligosaccharide 1 with traces of galacturonic acid and rhamnose, while the partial hydrolysis of the derived glycitol gave oligosaccharide 1 together with 2-O-(∝-D-galactopyranosyluronic acid)-L-rhamnitol. These two sugars were separated in solvent (E). The gas chromatography of the methanolysis products from the methylated tetrasaccharide gave major peaks with the retention times of the methylglycosides of 3,4-di-O-methyl-L-rhamnose and 2,3-di- and 2,3,4-tri-O-methyl-galacturonic acid. The same glycosides were identified in the methanolysate of the methylated glycitol together with 1,3,4,5-tetra-O-methylrhamnitol.

Oligosaccharide 4 (12 mg.) R_{GalA} = 0·4 (solvent C), 0·1 (solvent B), $[\alpha]_D$ = +82 (c, 0·44).

The sugar was chromatographically similar to O-(∝-D-galacto-pyranosyluronic acid)-(1 → 4)-O-(∝-D-galactopyranosyluronic acid)-(1 → 2)-L-rhamnose. Hydrolysis of the sugar gave galacturonic acid and rhamnose, whilst hydrolysis of derived glycitol gave galacturonic acid and rhamnitol. The sugar contained galacturonic acid and rhamnose residues in the molar ratio of 2:0·96. The methylated sugar was methanolysed and the products were examined by gas-liquid chromatography. Peaks were obtained which corresponded to the methyl glycosides of 3,4-di-O-methyl-L-rhamnose, 2,3-di- and 2,3,4-tri-O-methylgalacturonic acid.

(b) Acetolysis of leaf ammonium pectate

Leaf ammonium pectate (1·5 g.) was acetylated, as described previously, to give polysaccharide acetate (1·6 g.). The acetate was acetolysed (10 ml. of acetic acid + 10 ml. of acetic anhydride + 1 ml. of conc. sulphuric acid) for six days. The mixture of acetylated sugars after recovery from the acetolysis mixture, was dissolved in methanol and de-acetylated with methanolic barium methoxide (0·5 N; 25 ml.). The sugars (200 mg.) were recovered from the mixture and concentrated to a syrup.

The paper chromatography of the mixture showed the presence of the following sugar:-

Solvent (A):- galactose, glucose, arabinose, xylose, fucose, rhamnose and acidic oligosaccharides.

Solvent (B):- Acidic oligosaccharides with R_{GalA} = 0·13, 0·4 and 0·8.

The mixture was chromatographed on a diethylaminoethyl Sephadex A-25 column (5 g.; formate form). Elution with water (250 ml.) gave a mixture of neutral sugars (110 mg.). Paper chromatography of the mixture in solvent (A) showed the presence of galactose, glucose, arabinose, xylose, fucose, and rhamnose.

Elution of the column with 0·05 M-formic acid gave fraction I (20 mg.). Paper chromatography of the mixture in solvent (B) showed the presence of the following sugars:-

1. Oligosaccharide 1 (GalA1 → 2 Rha) — R_{GalA} = 0·8 — Orange colour with aniline oxalate.
2. Oligosaccharide 2 (GalA1 → 2Rha1→2Rha) — R_{GalA} = 0·4 — Yellow colour with aniline oxalate.
3. Traces of galacturonic acid.

Elution with 0·4 M-formic acid gave fraction II (17 mg.). Paper chromatography of the mixture in solvents (B) and (C) showed the presence of the following sugars:-

1. GalAl → 2 Rhal → 4GalAl → 2 Rha R_{GalA} = 0·55 (solvent C) and 0·13 (solvent B), gave an orange colour with aniline oxalate.

2. GalAl → 4GalA 0·2 (solvent B) (traces)

Elution with 0·5 M-formic acid gave fraction III (15 mg.) (chromatographically immobile). Hydrolysis of a portion of it gave predominantly galacturonic acid.

Preparation and methylation of carboxyl-reduced stem pectinic acid

1. Esterification of pectinic acid with ethylene oxide

Stem ammonium pectate (3 g.) was converted into pectinic acid by treatment with Amberlite resin IR-120 (H) and dispersed in water (300 ml.) giving a solution of pH 1·8. Ethylene oxide (50 ml.) was added and the solution was kept at room temperature for two weeks, the pH being occasionally noted. After two weeks the pH was seven and the esterification was complete. The excess of ethylene oxide was evaporated from the solution and the polysaccharide was precipitated with ethanol, washed with ethanol, redissolved in water and freeze-dried.

2. Acetylation of glycol ester

The glycol ester (1 g.) was dispersed in anhydrous formamide (25 ml.) with vigorous stirring for 24 hours. Pyridine (27 ml.) was added dropwise at about 40° and then acetic anhydride (18 ml.) was added dropwise to the mixture over a period of six hours, the temperature being maintained at 40° and vigorous stirring continued throughout. The solution was kept in the dark overnight and then poured dropwise into acidified ice-cold water, with vigorous stirring. The precipitated polysaccharide acetate was removed by centrifugation and washed with water a few times, dissolved in acetone and water was removed by azeotropic distillation with chloroform, under reduced pressure. The dried product was finally dissolved in chloroform and precipitated by light petroleum (b.p. 60-80)(10 vol.). The polysaccharide acetate (0·95 g.) was washed with light petroleum and dried over paraffin wax, in vacuo.

3. Reduction of acetate

The polysaccharide acetate (0·95 g.) was suspended in freshly distilled tetrahydrofuran (25 ml.). To this solution lithium borohydride (1 g.) in tetrahydrofuran (25 ml.) was added and the mixture was refluxed for three days with vigorous stirring. After cooling the solution, excess of borohydride was destroyed by carefully adding water. The solution was then neutralized with N-sulphuric acid. A small amount of residue (25 mg.) appeared and was removed at centrifuge, washed with water and dried by solvent exchange. On hydrolysis, the polysaccharide gave mainly galactose, and was not further examined. The supernatant solution and washings were concentrated to remove tetrahydrofuran and dialysed. The polysaccharide from the dialysate was precipitated with acetone (4 vol.), washed with acetone-water, redissolved in water and freeze-dried. Carboxyl-reduced pectic acid (0·2 g.) had $[\alpha]_D = +243^\circ$ (c, 0·25) and on hydrolysis the polysaccharide gave galactose with traces of arabinose and rhamnose.

4. Methylation of carboxyl-reduced pectic acid

Carboxyl-reduced pectic acid was first methylated by Haworth's method (88). The polysaccharide (170 mg.) was dissolved in water (5 ml.) and methyl sulphate (2 ml.) and sodium hydroxide solution (30%; 4 ml.) were added dropwise over a period of six hours with continuous stirring. The reaction flask was surrounded by an ice-bath and the reaction was carried out in an atmosphere of nitrogen. Four more additions of the reagents were made on four consecutive

days. On the sixth day the mixture was heated at 100° for one hour. After cooling, the solution was brought to pH 4 with dilute sulphuric acid and extracted with chloroform (6 x 50 ml.). The chloroform extracts were dried over sodium sulphate and concentrated to a syrup (150 mg.).

The partially methylated polysaccharide was next methylated by Purdie's method (89). The syrup (150 mg.) was dissolved in methyl iodide (20 ml.) and silver oxide (500 mg.) was added in five portions at hourly intervals with constant stirring and refluxing which was continued overnight. After cooling the mixture, silver residues were removed by filtration and extracted with hot chloroform. The supernatant solution and extracts were combined and concentrated to a non-reducing syrup. Three more methylations were carried out in the same way. The final chloroform solution of the methylated reduced polysaccharide was poured into light petroleum (b.p. 60-80) to give a white precipitate (70 mg.), which was removed by filtration, washed with light petroleum (b.p. 60-80) and dried over paraffin wax in *vacuo*; it had $[\alpha]_D$ + 180° (*c*, 0·4 in chloroform) (found: OMe, 41·2%).

A sample of the methylated polysaccharide was methanolysed with methanolic 4% hydrogen chloride. The products of methanolysis were examined by gas-liquid chromatography and gave peaks with the retention times of the methyl glycosides of the following sugars:- (In those cases where the relative retention time may be attributed to more than one sugar, the *T* value is given in parenthesis).

Sugars	Relative proportion	column (a) (125°)	T column (a) (150°)	column (c) (175°)
2,3,4-Me_3Xyl	+	0·33	0·45	(0·47)
		(0·48)	(0·53)	(0·58)
2,3,4-Me_3-Rha	+	0·41	0·49	(0·47)
2,3,5-Me_3-Ara	+ +	(0·48)	(0·53)	(0·58)
		0·62	(0·69)	(0·71)
2,3,4-Me_3-Fuc	+	0·66	(0·69)	0·67
3,4-Me_2Rha	4 +	0·83	0·87	0·98
2,3-Me_2Ara	+ +	1·09	(1·23)	1·46
		1·50	1·48	
2,3-Me_2-Fuc	+			1·08
3,4-Me_2-Xyl	+	1·02	1·08	1·24
		1·21	(1·23)	
2,3,4,6-Me_4-Gal	4 +	1·84	1·80	1·78
		1·98		
3-Me-Rha	+		(2·82)	3·37
2,3,6-Me_3Gal	major component		2·82	3·04
			3·68	3·70
			4·10	4·06
				4·50
3,6-Me_2Gal	6 +		8·31	8·37
			11·79	10·58
			13·52	13·16
2,6-Me_2Gal	6 +		9·64	9·71
			10·81	10·58
			15·53	14·40

Table XIII

Sodium acetate fractionation of alfalfa and citrus pectic acids

1. Alfalfa pectic acid

(a) De-esterification of pectinic acid (55):

N-Sodium hydroxide was added dropwise with vigorous stirring to alfalfa ammonium pectate (10 g.; from combined leaves and stems) at 0° until a pH12 was reached. The solution was kept at 0° for two hours, the pH being maintained at 12 ± 0·2 throughout. Aqueous 18% hydrochloric acid was then added dropwise to the clear solution, with vigorous stirring. When the precipitation was complete, the gel of the pectic acid was squeezed through a linen cloth and washed successively with acidified ethanol-water (3:2; containing 5 ml. of conc. hydrochloric acid per litre), ethanol-water (3:2), ethanol and ether. The polysaccharide was then dispersed in water and freeze-dried to give alfalfa pectic acid (8·3 g.). $[\alpha]_D$ = +239 (c 0·42), [found: uronic anhydride (potentiometric titration), 80·7%].

Fractionation of pectic acid with sodium acetate (20):

Pectic acid (8·0 g.) was dissolved in water (800 ml.) containing N-sodium hydroxide (36 ml.), to give a solution having pH 6·5. 2M-Sodium acetate solution (50 ml.) was added with vigorous stirring and the mixture was kept at 0° for 18 hours. The precipitate was then removed at the centrifuge and washed with 0·12 M-sodium acetate solution. The sodium pectate was redissolved

in water and precipitated as free pectic acid with acidified ethanol (1 vol.; containing 4% acetic acid). The precipitate removed at centrifuge, washed free of acetic acid with ethanol-water (1:1), redissolved in water and freeze-dried. The pectic acid (0·45 g.) had $[\alpha]_D$ = +268° (c, 0·45; as sodium pectate) [found: uronic anhydride (titration), 92%].

A second fraction (1·1 g.) was obtained by the addition of further 2 M-sodium acetate solution (10 ml.) to the mother liquors from the first fractionation, and isolating the precipitated polysaccharide as in the first case. This fraction had $[\alpha]_D$ = +255° (c, 0·52; as sodium pectate) [found: uronic anhydride (titration), 87%].

A third fraction (0·7 g.) having $[\alpha]_D$ = +248° (c, 0·4; as sodium pectate) [found: uronic anhydride (titration), 84%] was obtained by a further addition of 2 M-sodium acetate (20 ml.) to the mother liquors from the second fractionation.

A further addition of 2 M-sodium acetate (20 ml.) to the mother liquors from the third fractionation gave a fourth fraction (1·38 g.) having $[\alpha]_D$ = +244° (c, 0·45; as sodium pectate) [found: uronic anhydride (titration), 83%].

No further precipitates were obtained by the addition of more sodium acetate, the residual polysaccharide in the solution was precipitated with ethanol (1 vol.), which furnished a fifth fraction (2·3 g.) having $[\alpha]_D$ = +236 (c, 0·36; as sodium pectate) [found: uronic anhydride (titration), 78·5%]

All the fractions were examined by moving boundary electrophoresis, in pyridine-acetic acid buffers (pH 4·5) and gave single

peaks with the mobilities quoted in table IV (p.49). Samples of polysaccharide fractions were hydrolysed in N-sulphuric acid and chromatography of the hydrolysates in solvents (A) and (B) showed the presence of galacturonic acid as the major sugar component together with small amounts of the neutral sugars which were present in the original pectinic acid.

2. Fractionation of citrus pectic acid

Citrus pectin (20 g.), extracted with cold water from lemon peel (49), in water (1 litre) was adsorbed on a diethylaminoethyl-Sephadex (A-50) column (100 g.; 9·5 x 75 cm.; formate form) and the column was eluted with water (20 l.) till the eluate gave a negative test to the phenol-sulphuric acid reaction. The water eluate was concentrated and the polysaccharide was precipitated with ethanol, redissolved in water and freeze-dried. The dried polysaccharide (0·8 g.) had $[\alpha]_D$ = +38 (c, 0·4) [found: uronic anhydride (decarboxylation), 10%]

The column was then eluted with 0·2 N-formic acid (16 l.). The eluate was dialysed for 36 hours. After adjusting the pH to 4·5 with potassium acetate, the solution was concentrated to a small volume. The polysaccharide was precipitated with ethanol containing 4% acetic acid, removed at the centrifuge, washed free of acetic acid with ethanol, redissolved in water and freeze-dried. This polysaccharide fraction (0·4 g.) had $[\alpha]_D$ = +76 (c, 0·25) [found: uronic anhydride, 27%]. The column was then eluted with N-formic acid (30 l.) and the polysaccharide (18 g.), recovered from the eluate as in the previous case had $[\alpha]_D$ = +220 (c, 0·25)

[found: uronic anhydride (the carbazole and titration methods), 75%].

The major pectinic acid fraction (20 g.) was de-esterified, as in the case of alfalfa pectinic acid, to give pectic acid (15·3 g.) having $[\alpha]_D = +242^\circ$ (c, 0·4, as sodium pectate) [found: uronic anhydride, 84%]. In addition, acid soluble polysaccharides (0·4 g.) in the filterate form the pectic acid precipitation were recovered by complete precipitation with ethanol. The polysaccharide fraction had $[\alpha]_D = +100^\circ$ (c, 0·3) [found: uronic anhydride (titration) 36%], and on moving boundary electrophoresis gave four peaks. Hydrolysis of the polysaccharide gave galacturonic acid, galactose, glucose, arabinose, rhamnose and xylose.

Pectic acid (14 g.) in water (1400 ml.) was fractionated, as in the case of alfalfa pectic acid, to give four fractions at 0·12 M, 0·14 M, 0·18 M, and 0·22 M-sodium acetate concentrations and a fifth fraction was obtained by complete precipitation of the residual polysaccharide in solution, with ethanol. All the fractions were examined by moving boundary electrophoresis, specific rotations of their sodium salts were measured and uronic anhydride content was determined by potentiometric titration. The following fractions were obtained.

1. Pectic acid fraction I (0·7 g.) precipitated at 0·12 M-sodium acetate, $[\alpha]_D = +288^\circ$ (c, 0·25) (uronic anhydride, 94%).
2. Pectic acid fraction II (3·3 g.) precipitated at 0·14 M-sodium acetate, $[\alpha]_D = +265^\circ$ (c, 0·5) (uronic anhydride, 88%).
3. Pectic acid fraction III (5·6 g.) precipitated at 0·18 M-sodium acetate, $[\alpha]_D = +257^\circ$ (c, 0·42) (uronic anhydride, 85%).

4. Pectic acid fraction IV (1·7 g.) precipitated at 0·22 M-sodium acetate, $[\alpha]_D$ = +240° (c, 0·25) (uronic anhydride, 82%).

5. Pectic acid fraction V (1·2 g.) precipitated with ethanol, $[\alpha]_D$ = +186° (c, 0·29) (uronic anhydride, 68%).

All the fractions on hydrolysis gave galacturonic acid as major component with small amounts of neutral sugars, i.e., arabinose, galactose, rhamnose, fucose and xylose.

Methylation of citrus pectic acid (fraction III)

The polysaccharide fraction III (3 g.) obtained from sodium acetate fractionation of citrus pectic acid was dissolved in water (50 ml.) and methylated with sodium hydroxide solution (30%; 40 ml.) and methyl sulphate (20 ml.) in an atmosphere of nitrogen, the reaction flask being surrounded by an ice-bath, in the usual way. Five additions of the reagents were made on five consecutive days. The final mixture was heated on a boiling water bath for an hour. On cooling the precipitated sodium sulphate was filtered off and washed with water. The filterate and washings were dialysed, concentrated to a small volume and freeze-dried. The freeze-dried partially methylated polysaccharide was converted to silver salt and then methylated (four times) with methyl iodide and silver oxide, as described in the methylation of carboxyl-reduced alfalfa pectic acid. The final chloroform solution of the methylated pectic acid was poured into light petroleum (b.p. 60-80) (10 vol.). The precipitated methylated polysaccharide (0·26 g.) was filtered off, washed with light petroleum and dried over paraffin wax, in *vacuo*. It had $[\alpha]_D$ = +135 (*c*, 0·2 in chloroform) [found: OMe, 38%].

A sample of methylated polysaccharide was methanolysed and examined by gas-liquid chromatography. Peaks with the retention times of the methyl glycosides of the following sugars were obtained:-

(In those cases where the relative retention time may be attributed to more than one sugar, the *T* value is given in parenthesis).

Sugars	T		
	column (a) (125°)	column (a) (150°)	column (c) (175°)
2,3,4-tri-O-methyl-D-xylose	0·35	(0·44)	(0·47)
	(0·46)	(0·49)	(0·55)
2,3,4-tri-O-methyl-L-rhamnose	0·40	0·44	(0·47)
2,3,5-tri-O-methyl-L-arabinose	(0·46)	(0·49)	(0·55)
	0·62	(0·65)	(0·72)
2,3,4-tri-O-methyl-L-fucose	0·66	(0·65)	(0·72)
3,4-di-O-methyl-L-rhamnose	0·82	(0·88)	1·03
2,3-di-O-methyl-L-fucose	0·9	(0·88)	1·06
	1·20	1·12	1·23
	1·31		
2,3-di-O-methyl-L-arabinose	1·09	1·19	1·47
	1·48	1·45	(1·76)
	1·58		
2,3,4,6-tetra-O-methyl-D-galactose	1·85	1·77	1·76
	1·98		
3-O-methyl-L-rhamnose		(2·76)	3·39
2,3,4-tri-O-methyl-D-galactose		2·76	3·06
		3·57	4·10
		(4·25)	4·53
2,3-di-O-methyl-D-galacturonic acid		4·25	4·95
2,3,4-tri-O-methyl-D-galacturonic acid		6·26	6·98

Table XIV

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